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*DISTRIBUTION AND MOTION OF INTERSTELLAR HYDROGEN IN THE GALACTIC SYSTEM WITH PARTICULAR REFERENCE TO THE REGION WITHIN 3 KILOPARSECS OF THE CENTER**

By G. W. Rougoor and J. H. Oort

LEIDEN OBSERVATORY

Distribution of the Interstellar Gas.—Observations of the 21-cm emission of the hydrogen atoms in the interstellar medium have enabled astronomers to obtain a general survey of the distribution of interstellar gas throughout the Galactic System. The main features of this distribution may be described as follows. The gas is strongly concentrated into a very thin layer. The thickness as given by the distance between the planes where the density has dropped to half the maximum density averages about 220 pc,† which is of the order of $^1/_{100}$ of the disk's diameter. It is only near its center that the disk becomes considerably thinner. Over most of its extent the hydrogen layer is surprisingly flat: within 6 kpc from the center the points of maximum density deviate nowhere more than 75 pc from the mean plane, except in a few small areas. It is only in the thinner, outer regions of the Galactic System that the layer begins to deviate from the plane defined by the principal part of the disk. These deviations have a systematic character, the layer tipping up on one side and down on the opposite side; the greatest deviations from the plane are 600 and 800 pc, and occur at distances of about 13 kpc from the center. This interesting phenomenon was first found by Kerr, Hindman, and Carpenter.[1]

The gas, as is well known, is composed mostly of hydrogen and helium atoms, with a small admixture of heavier elements. Most of the hydrogen is neutral; the ionized part is probably only of the order of 5 per cent.

There must be *some* gas far outside this main disk, connected with the magnetic fields which seem to play an essential part in the "radio corona" surrounding the Galactic System. So far, this gas has not been directly observable. In a very indirect manner it has been estimated that its density may be of the order of 0.01 atom/cm^3 and its temperature 10^6 °K. Both numbers are extremely uncertain.

The distribution of the gas in the disk is very far from uniform. It is concentrated in clouds of widely varying sizes, densities, and forms, which have a tendency to form huge agglomerations and are more or less arranged in long bands winding through large parts of the Galactic System. The general distribution may be seen in Figure 1. Although the evidence obtained from the 21-cm observations is not quite complete enough to obtain a coherent map of these bands, the close analogy between the Galactic System and spiral galaxies of the so-called Sb type, renders it rather probable that the bands of gas in our galaxy form a spiral pattern. The spiral arms are evidently very irregular, with more or less sudden breaks,

subsidiary arms, etc.; irregularities quite like those found abundantly in all spiral systems.

The arms in the Galactic System are about 2 kpc apart; a radius vector from the center would cross four or five main arms beyond 3 kpc from the center.

The 21-cm observations have greatly extended our knowledge concerning the rotation of the System. The angular velocity is found to increase strongly towards

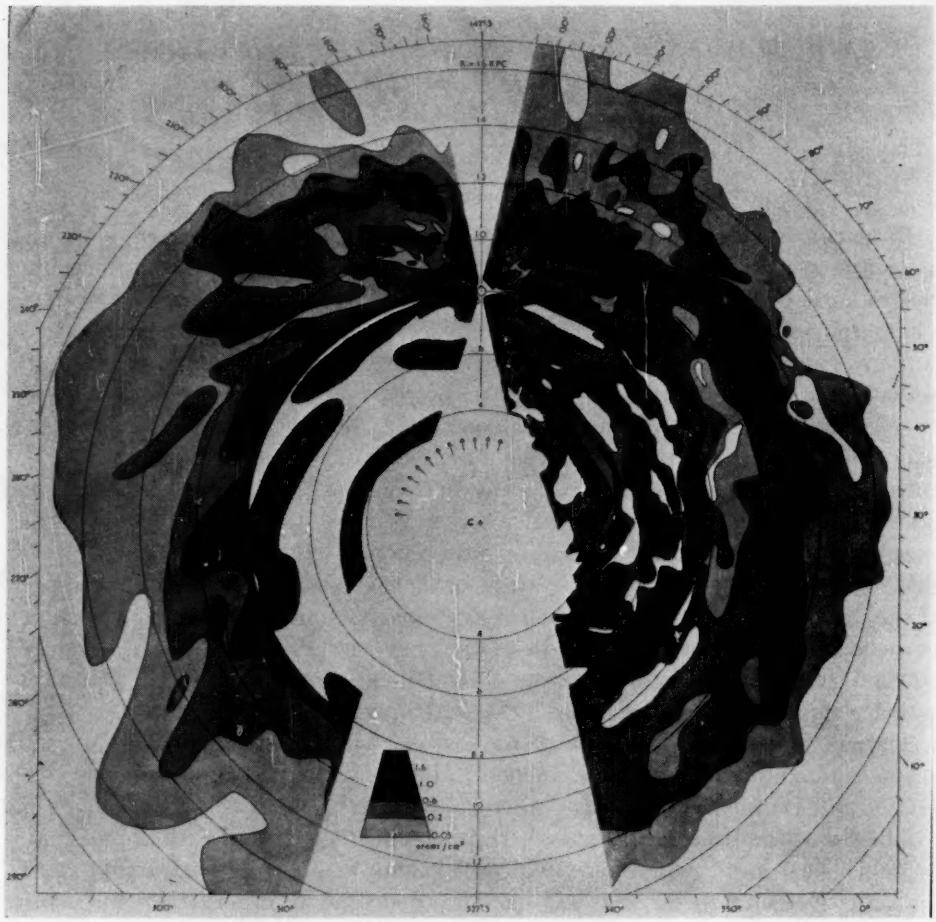

FIG. 1.—Distribution of neutral hydrogen in the galactic plane after observations made in Australia and in the Netherlands (from Oort, Kerr and Westerhout[10]). The center of the Galactic System is at *C*, the position of the sun has been marked by a small circle indicated by *S*. The hydrogen in the central part has not been drawn in. The numbers on the sides indicate galactic longitudes, on the old system. Distances from the center are indicated in kiloparsecs. Blank spaces between 311° and 340° and between 135° and 160° are due to the fact that these parts cannot be "resolved" because of the smallness of differential rotation in these longitude intervals.

the center. At 4 kpc it is about twice that near the sun, which lies at 8.2 kpc from the center. In the absence of some form of gas transport between the arms, the spirals would seem to have to wind up, so as to be practically effaced in a time brief compared to the age of the Galactic System. As we shall see below, the angular velocities in the central region are still considerably higher than at 4 kpc. At 500

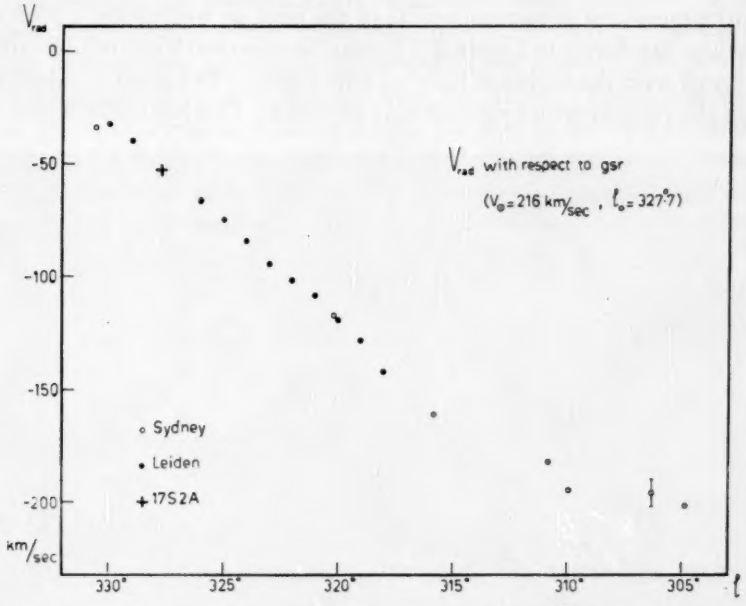

FIG. 2.—Radial velocity of the 3-kpc arm at different longitudes. The longitudes are on the old system. The cross gives the velocity of the absorption line due to the radio source Sagittarius A.

pc from the center they are roughly twenty times that near the sun, at 100 pc even a hundred times (cf. Fig. 8). Here the problem of the permanence of the spiral arms becomes still much more difficult.

Expansional Motions in the Central Part of the Galactic System.—In the region farther than 4 kpc from the center nothing has, so far, been found to indicate large-scale deviations from circular motion. But in the central part, which we shall define roughly as the region within 3 kpc of the center, we observe quite unexpected motions. The 21-cm observations indicate a rather dense arm situated at about 3 kpc from the center and passing between the sun and this center. The arm is very well defined, and more homogeneous in density as well as velocity than any of the outer arms. It takes part in the rotation of the System at a velocity of probably about 200 km/sec. The unexpected feature, which was discovered by van Woerden *et al.*[2] in 1957, is that the entire arm appears to move out radially, away from the center, at a velocity which, at the point where it passes in front of the center, is −53 km/sec. At this point it is seen in absorption against the radio source Sagittarius A, which forms the nucleus of the Galactic System. The emis-

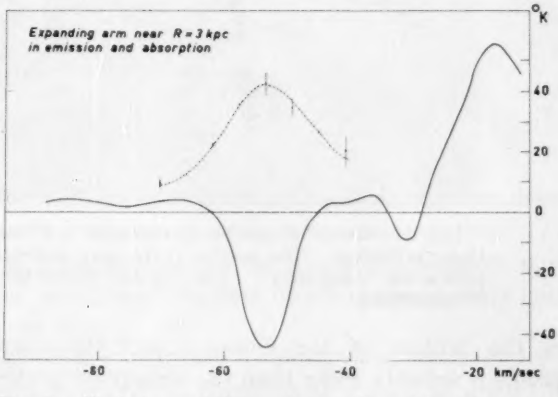

FIG. 3.—Emission and absorption profiles of the 3-kpc arm.

sion velocities measured in various points of the arm, as well as the velocity of the absorption line, are shown in Figure 2. It may be observed that the absorption line fits in very well with the emission lines on either side. The great regularity of the variation of the velocity with longitude may be noted. This regularity is also striking

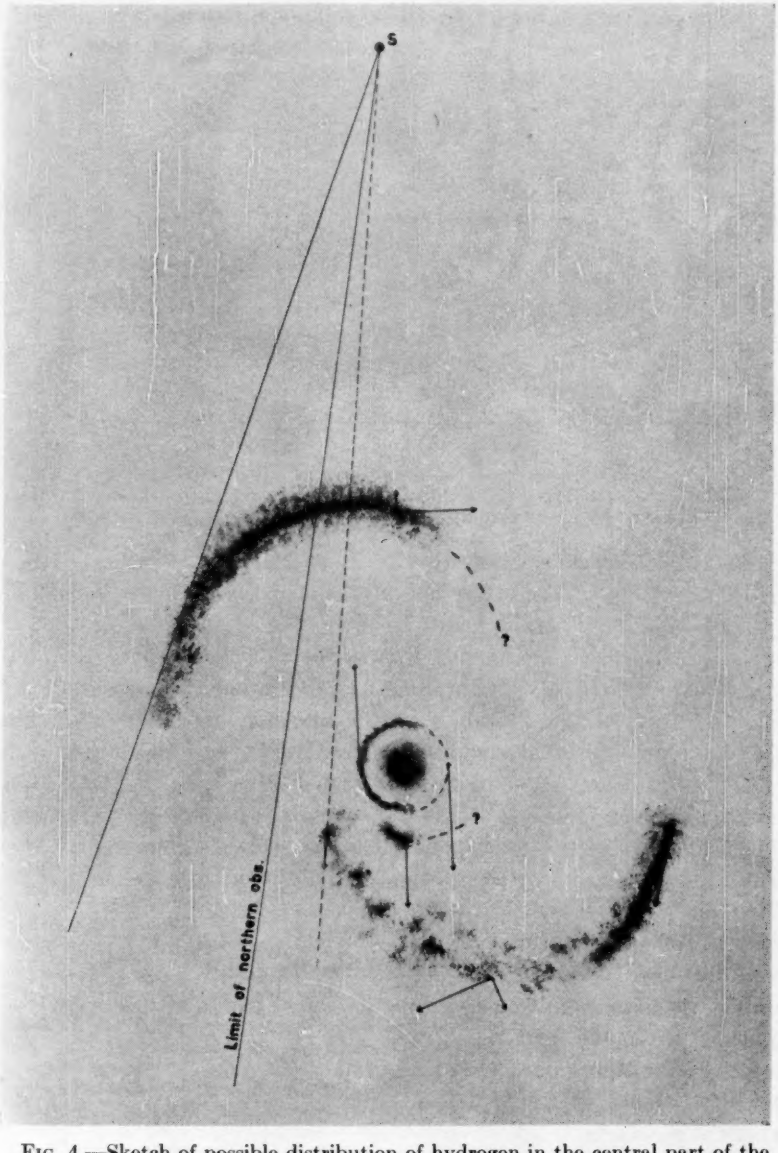

FIG. 4.—Sketch of possible distribution of hydrogen in the central part of the Galactic System. The position of the sun is indicated by S. The arm in the upper part is the "3-kpc arm." The ring and disk in the nuclear region do not seem to be expanding.

in the profiles of the emission and the absorption (Fig. 3). The emission profile is sensibly wider than the absorption profile, the half-widths being 15.8 and 10.6 km/sec, respectively. The cause of this difference is still unknown. The

situation of the arm has very tentatively been sketched in Figure 4. The observations made in Sydney have been interpreted to indicate that it becomes tangential to the line of sight near galactic longitude 303° (old system), which would correspond to a distance of roughly $3^1/_2$ kpc from the center. For this reason the arm has been drawn at a radius of about 3 kpc, and will be referred to as the "3-kpc arm." It should be emphasized, however, that the conclusion as to where it becomes tangential is quite uncertain. Its distance at the point where it crosses the center is still more uncertain. It is quite possible that the arm spirals rapidly

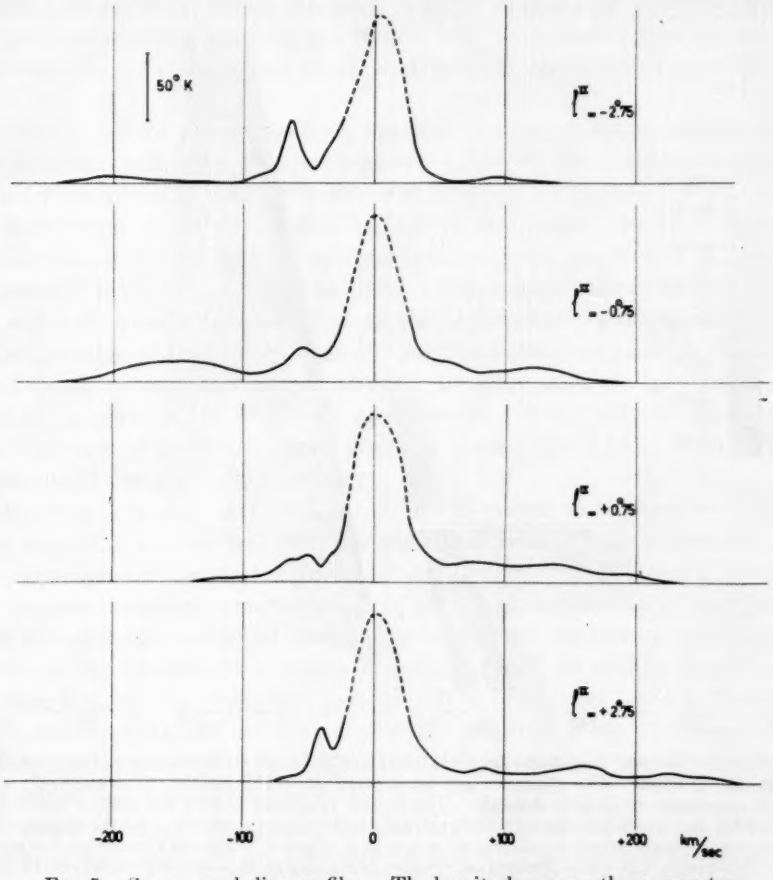

FIG. 5.—Some sample line profiles. The longitudes are on the new system, counted from the direction of the center as zeropoint. The principal maximum, which is almost entirely due to hydrogen outside the central region, and which has not been measured in the present program, is indicated by a dotted curve.

inward, and that at this point it is rather closer to the center than has been drawn in Figure 4. The part shown in the picture is the only part we can observe. The arm is likely to continue on both sides beyond this stretch, but it becomes indistinguishable because its velocity coincides with that of other spiral arms much closer to, or much farther from the sun.

The "expanding arm" is clearly shown in Figure 6, which gives the intensity of the hydrogen radiation, expressed in the usual temperature scale,‡ as a function

of radial velocity and galactic longitude. The principal maximum has been left out. This contains almost entirely matter closer to the sun, or gas beyond the central region. A few samples of the line profiles that were used to construct this diagram are shown in Figure 5. The "3-kpc arm" is shown in Figure 5 in the secondary maxima and in Figure 6 by the narrow ridge of high temperature to the left of the broad central maximum which has been left blank. The interruption of the ridge near the longitude of the center and the depression of the feature in the profiles

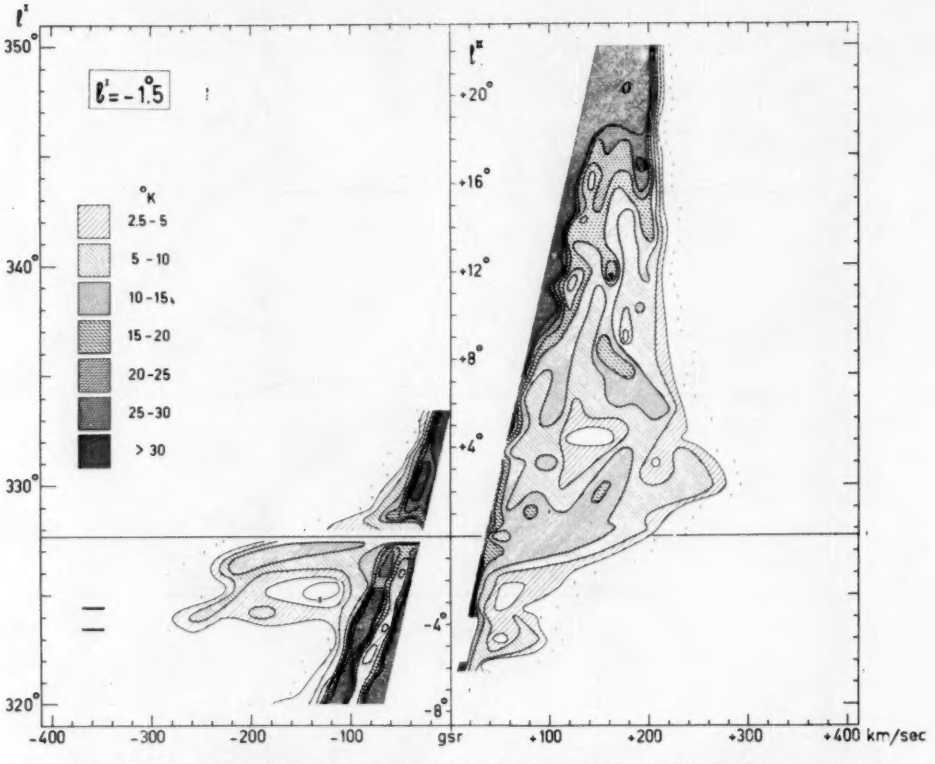

FIG. 6.—Contours of brightness temperature‡ in the 21-cm emission line in the central region of the Galactic System. The contours are for a plane at $-1°.5$ latitude (old system); this is the plane of maximum hydrogen density. The radial velocities shown are values which have been corrected for the motion of the sun with respect to the galactic center. At the negative velocities the curves have been interrupted over an interval of about $0°.5$ around the center, because, on the scale of this graph, the steep absorption profiles occurring in this interval could not be drawn adequately.

for $l^{II} = \pm 0.°75$§ are only apparent, and due to the absorption phenomenon described above.

From a further inspection of Figure 6 and of similar diagrams for latitudes $-0°.5$, $-1°.0$, and $-2°.0$, we see that the 3-kpc arm is not the only case of matter streaming away from the center. If one observes precisely at the longitude of the center, one finds quite considerable intensities between velocities of +50 and +200 km/sec. Between +100 and +200 km/sec there is no appreciable absorption of the radiation of Sagittarius A. This means that the bulk of this gas must lie behind the center and must therefore move away from it with velocities of the order mentioned.

If we now consider the lower longitudes, we see that there is a certain amount of hydrogen moving with radial velocities above +30 km/sec, up to a limit varying from about +100 to +120 km/sec. The rotation of the system is such that matter observed in longitudes lower than that of the center should approach us if it were moving in circular orbits, and thus have negative velocities. As it is practically certain that in this case we are dealing with matter which is not only close to the center in angular measure, but is actually in the region within about 3 kpc of the center, the rotational components projected on the line of sight are likely to be quite high. In order to obtain the high positive line-of-sight velocities observed for the gas concentration between $-1.°5$ and $-5.°5$ longitude§ this gas must presumably have radial components of at least 200 km/sec directed away from the center.

Although we cannot prove this from the observations, it appears probable that also the hydrogen with high positive velocities in longitudes *larger* than that of the center has a considerable velocity component directed away from the center.

The Distribution of the Gas within 3.5 kpc of the Center.—In view of the evident, enormous deviations from circular motion occurring within 20° of the center, it is impossible to use the observed Doppler shifts to locate the hydrogen in space. We *can* say with some confidence that all the high-velocity gas observed in these longitudes pertains to the central region. Furthermore, it is possible to conclude that some parts are virtually devoid of gas. In other cases we have made an attempt to locate some of the matter by assuming that it is partly arranged along a spiral, which may even be connected near the outer edge of the "central region" with a reasonably well-defined spiral arm.

It is clear that any sketch of the distribution of matter in the central part, such as shown in Figure 4, must be extremely uncertain and, at best, schematic. This holds in particular for the matter drawn behind the center. It is not at all certain that the various detached agglomerations of gas which we observe at high positive velocities are arranged in a spiral arm, nor whether they connect up with the strong arm drawn in the lower right corner. This latter itself, as well as the 3-kpc arm in the upper left, appear to be well defined. It is likely that some of the matter with high positive velocities between 4° and 22° longitude lies in the space between the arm beyond the center and the 3-kpc arm. We have not drawn it because there is no indication *where* to put it.

On the other hand it is practically certain that the space below the 3-kpc arm, between the dotted line at $l^{II} = -6°$ and the line marked "limit of northern obs." at $l^{II} = -10°$, is virtually empty.

Although it appears plausible to assume that all the large deviations from rotational motion are directed away from the center, as in the 3-kpc arm, even this is not certain. From the emission velocities themselves we cannot distinguish between radial motions towards or away from the center. For the present, however, we shall assume as a working hypothesis that the motions are all outward.

A remarkable feature of the central region is the great difference in appearance between the side of positive velocities and that of negative velocities. If we abstract from the wing of high negative velocities between 0° and −4° longitude which, as we shall see below, is an entirely different feature pertaining to a region closer to the center than we are now discussing, we find that all the gas on the side of negative

velocities is concentrated in a well-ordered and continuous structure, the 3-kpc arm. But the gas clouds having *positive* velocities show hardly any order at all. There is apparently a fairly important asymmetry in this central region. Yet, the total amounts of gas appear to be approximately the same in the two halves.

It should be pointed out that, notwithstanding their sometimes very large deviations from circular motions, all features observed in the central region lie very close to the average galactic plane. There are no indications of considerable motions perpendicular to this plane.

Disk and Ring Structure in the Nuclear Part—The Variation of Circular Velocity with Distance from the Center.—A radical change in the distribution and motion of the interstellar gas seems to occur at $R = 600$ pc. The character of this change is displayed most clearly at the side of the negative velocities. Perhaps the most striking feature in Figure 6 is the wing towards high negative velocities beginning at longitude $-4°$ and extending to the longitude of the center. The fact that no

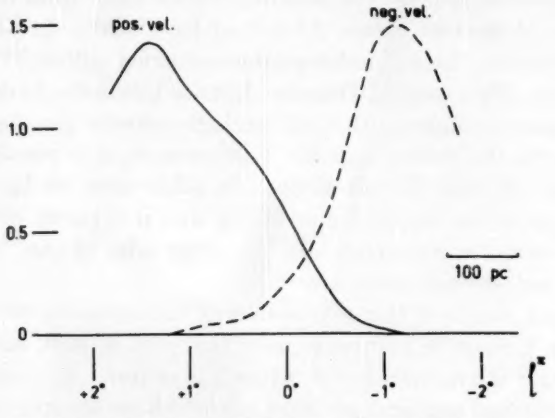

FIG. 7.—Numbers of hydrogen atoms per cm² with radial velocities between 150 and 210 km/sec (after correction for the motion of the sun relative to the galactic center). The numbers refer to latitude zero on the new system; abscissae are longitudes in the same system. Ordinates are in units of 10^{21} atoms.

such high negative velocities are found in any other part of the longitude interval investigated|| and the exact co-incidence of the upper longitude limit with the direction of the center, suggest strongly that the wing is due to matter situated close to the center.

When we now look at the postive velocities, we notice that for these there is a very similar feature at longitudes beyond that of the center. Here, however, it is superimposed upon an extremely irregular background of high-velocity matter making up the very irregular arms shown in the picture. That there is something real superimposed on this background is indicated by the curves in Figure 7 which were obtained from special measurements of high accuracy made at each quarter of a degree from $-2°$ to $+2°$ longitude. These show that the number of atoms of very high positive velocity drops steeply near the longitude of the center, in very much the same way as the high negative velocities. Also, the total numbers of atoms in the velocity range considered is almost exactly the same for positive and negative velocities.

The most plausible interpretation of these new features is that they are due to a disk of hydrogen extending to about 600 pc from the center and rotating at high velocity. A more detailed analysis of the line profiles for the negative velocities—which are uncontaminated by foreground or background radiation—indicates that there is a ring of matter with rather sharp inner and outer boundaries at radii R of about 500 and 590 pc. The density in the ring is 1 atom per cm^3. There appears to be a practically empty space within the ring, the density only becoming appreciable again at $R = 300$ or 350 pc. It then rises gradually to about 3 atoms per cm^3 at $R = 100$ pc. Still further inside the density must increase very strongly. The distribution is shown schematically in Figure 4. In the innermost region the interpretation of the observations becomes complicated because of the absorption phenomena caused by the radio source Sagittarius A. Drake in Green Bank[3] has recently shown that the nucleus of this radio source contains two very dense patches about 15 pc from the galactic center. If one assumes that these are regions of ionized hydrogen, the masses would be of the order of 50 000 solar masses. Smeared out over a sphere of 20 pc radius this would correspond with a density of about hundred protons per cm^3. It has been suggested by Woltjer that the ionization in the emission regions would be caused by the ultraviolet radiation of the population II stars rather than by O-type supergiants, which could hardly be sufficiently frequent to furnish a lasting source of energy. The population II stars may be sufficiently dense in the central region to explain this radiation.

If a radio telescope with a beamwidth of 0.°56, like the one used for the present study, is pointed towards the center, the inner parts of the rotating disk are seen in absorption against the wings of Sagittarius A. The absorption has been measured up to velocities of a little over 100 km/sec. It is present at negative as well as at positive velocities. Some sample curves are shown in Figure 9.

There is no sign of any expansional motion in this "nuclear" disk and ring, neither in the emission observed at negative velocities nor in the absorption profiles. There appears to be only rotation. The rotational velocities are quite high. In the ring, at 550 pc, it is about 265 km/sec. Rough estimates from the observed line profiles give velocities of 220 km/sec at $R = 260$ pc, 190 km/sec at $R = 140$ pc and about 180 km/sec at $R = 70$ pc. Assuming, tentatively, that the motions in the ring and disk are governed mainly by gravitation and that these values give the circular velocities, we can derive the concentration of mass density in the nuclear region of the Galactic System. It is well known that in most galaxies which contain a fairly strong population II there is a strong increase in light-density right up to the center. In the Galactic System, where the entire central region is hidden by very opaque dark material, this cannot be observed. But it can be seen quite well in the Andromeda nebula, a galaxy bearing probably a fairly strong resemblance to our own galaxy. In this nebula the increase of light continues to a distance of only a few parsecs from the center. Baade states that the nucleus has diameters of $2.''5 \times 1.''5$, corresponding with 7×4 pc. It is interesting to compare the rotational velocities observed in the Galactic System with those found for the inner part of the Andromeda nebula, on the assumption that the distribution of light is the same as that of the mass, and that the mass-to-light ratio is 20.[4] The space distribution of the light up to about 100″, or 300 pc, was derived from plates taken for this purpose in 1932 with the 69-inch Perkins reflector in Delaware, Ohio. For

the region up to about 3 kpc use was made of photometric measures by Fricke[5] and by Redman and Shirley.[6] A rough computation being sufficient for our present purpose, the measures along the major axis and the least obscured, southeastern, half of the minor axis were averaged, and the region considered was treated as if it had spherical symmetry.

The agreement between the circular motions thus computed for the Andromeda

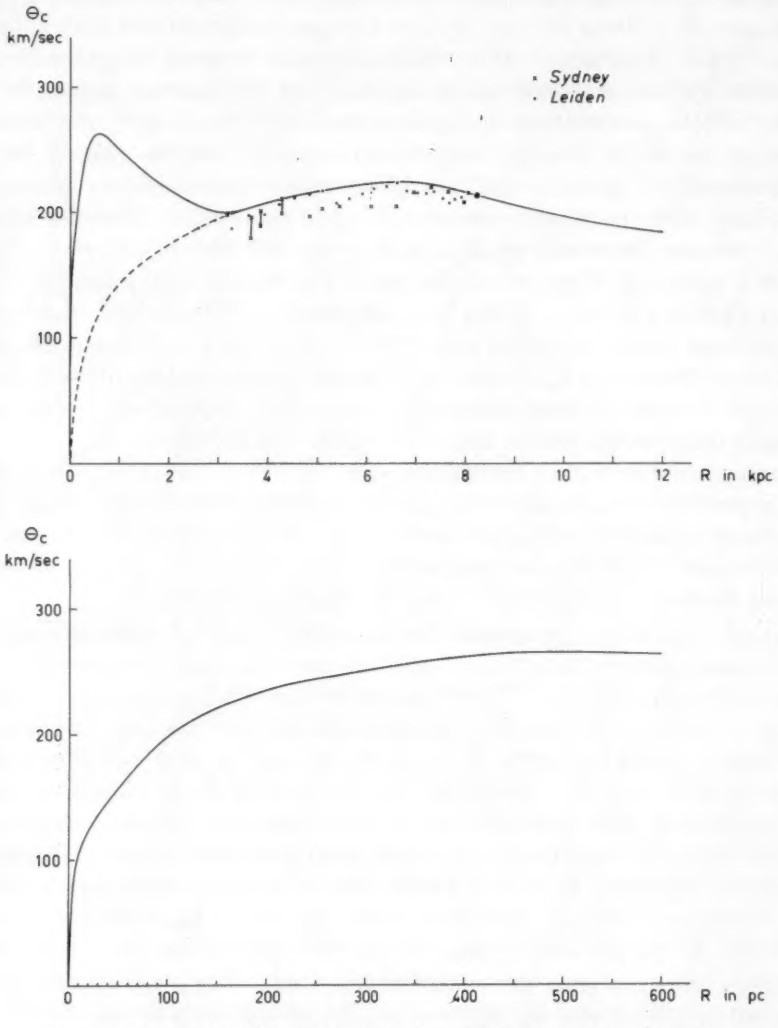

FIG. 8.—The variation of circular velocity with distance from the center.

nebula and the rotational velocities measured in the central disk and ring and between 3 and 4 kpc in the Galactic System appeared to be quite good, except that a reduction factor of 0.79 had to be applied to the Andromeda nebula results. This reduction factor may change slightly for distances greater than 600 pc from the center. The rough Andromeda-nebula data have been used to extend to observed parts of the rotation curve for the Galactic System into the region within $R = 100$ pc, where it could not be directly measured, and over the interval from 600 to about

3,000 pc, where the unexplained expansional motions prevent any direct estimates of the circular velocities. The resulting circular velocities are shown graphically in Figure 8. It should be stressed that this is only a provisional result, which will be subject to revision after the final reductions have been made. The part beyond $R = 3$ kpc is based on earlier 21-cm data obtained in Kootwijk (Netherlands) and in Sydney. The following table summarizes some of the pertinent data derived from this curve and from the preceding discussion. They are compared with results at 3 and 8.2 kpc derived from other data. The mass density is seen to rise to 1,000 times that near the sun around $R = 90$ pc. At $R = 10$ pc it runs up to roughly 24,000 times this density. The density is almost entirely due to *stars:* within 500 pc of the center the interstellar hydrogen contributes only about $^1/_{400}$ to the total mass. The periods of revolution at the various distances considered are shown in the last column.

R (parsecs)	Mass within R (10^9 suns)	Density: stars (10^{-22} g/cm^3)	Density: gas (at/cm^3)	Density: gas (10^{-22} g/cm^3)	T (10^6 years)
10	0.03	24000	1000:	100:	0.5
20	0.07	7800	100:	10:	1.0
100	0.92	710	3	0.5	3.1
500	8.3	42	1	0.2	12
3000	16	6.9	0.4	0.1	96
8200	60	1.0	1	0.2	230

A few data may be added concerning the distribution in the direction perpendicular to the galactic plane, which we shall call the z direction. In the 3-kpc arm the distance between the two planes where the density drops to half its maximum value is 1.°5, or 120 pc, which is about half the thickness of the layer in the general region between 5 and 10 kpc from the center. Roughly the same linear thickness is found for the aggregate of the matter with high positive velocities.

In the nuclear disk the half-density thickness decreases to about 80 pc. The derivative $\delta K_z/\delta z$, where K_z is the z component of the gravitational force, becomes so large when we come very close to the center, that within $R = 50$ pc the internal velocities must become of the order of 100 km/sec in one co-ordinate to explain the observed thickness.

Replenishment of Gas in the Central Disk and Possible Cause of the Outward Flow.— We are still far from an understanding of the behavior of the gas in the central region. We consider, first, the problem of the replenishment of the gas streaming outward through the thin galactic layer. A very rough estimate gives that per year a mass of hydrogen equal to between one and two solar masses moves outward through a cylinder of 2.5 kpc radius. At this rate the central disk would be depleted of its hydrogen in a time of the order of 10^7, or, at most, 10^8 years. This is so short compared to the age of the Galactic System that we have to conclude that the gas must be replenished in some way. Unless we assume that it is produced from matter in some entirely unknown state, such as has been proposed by Ambartsumian, there are only two possible sources: the stars in this region and the galactic corona. If one computes, on the basis of current theories of stellar evolution, the amount of gas that could be expelled by the population II stars in the central region, one arrives at a figure which is about 50 times too low. We must therefore probably consider the galactic corona as the source of replenishment. Rough estimates indicate that, with a plausible coronal density, just about enough gas may condense into the

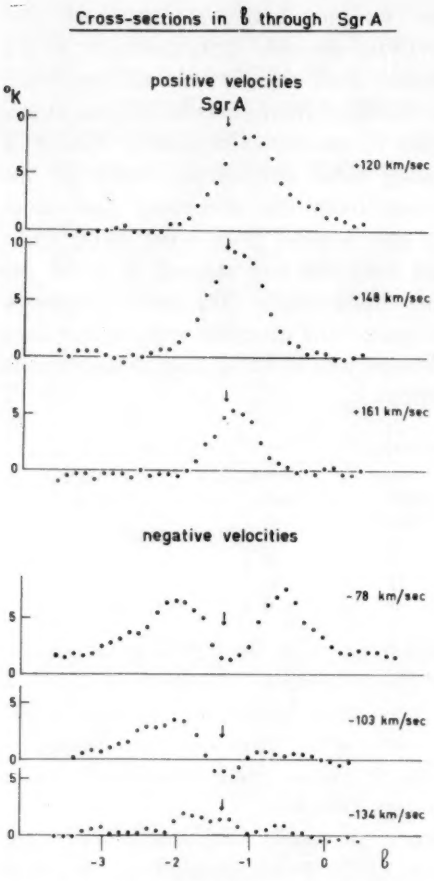

FIG. 9.—Sample distributions in latitude at the longitude of the galactic center. The absorption shown in the lower half is due to absorption of the radiation from the wings of the radio source Sagittarius A in the outer parts of the beam of the telescope. The absorption presumably takes place in the nuclear disk, mainly at distances between 10 and 50 pc from the center. It extends from about −75 to −120 km/sec; the absorptions found at lower negative velocities, down to −40 km/sec, are due to the 3-kpc arm. At *positive* velocities absorption due to the nuclear disk has likewise been observed, but here the measurements are more difficult and the results more uncertain. At higher positive velocities the line appears in emission, as shown in the upper part of the diagram. It shows matter behind the center, moving away from it at high velocities.

cool disk to make up for the loss by the matter streaming away in the plane of the disk.

Work on the theoretical side of the problems discussed is only in its very beginning. The cause of the spiral phenomenon in general is still unknown and it is also unknown how the spiral pattern is maintained against the effects of differential rotation. Almost the only thing we *can* understand is why the galactic layer is as thin as it is found to be. In addition, an interesting theory has been developed by Kahn and Woltjer[7] to account for the systematic bending of the outer edges of the layer as a consequence of the motion of the Galactic System relative to an intergalactic medium.

No theory has yet been developed to explain the systematic outward motions in the central region. Nor has it been possible to understand the existence of the spiral-like features which may be seen in the corresponding region of the Andromeda nebula, and which probably exist likewise in the central region of the Galactic System. With the large differential circular velocities and the short times of revolution in the central parts of these galaxies such features would rapidly be effaced if they were governed by gravitational forces only. The only likely other force which could influence these structures and motions would seem to be a hydromagnetic force. But it is still unknown in what manner it would push the gas outward and contribute to the observed structures.

As regards the small nuclear disk, it has been suggested by Woltjer that the magnetic fields in this disk, which are responsible for the extended non-thermal component of Sagittarius A, would have circular symmetry and form a closed unit.

The observations on the spiral structure outside the central region were partly made at Kootwijk in the Netherlands, using a 7 1/2-meter giant Würzburg,[8] and partly in Sydney, with a 11-meter meridian radio telescope.[9] A general account may be found in an article by Oort, Kerr, and Westerhout;[10] a more detailed survey

article has recently been published by Elwert.[11] The observations on the central region were almost entirely made with the 25-meter radiotelescope of the radio astronomy observatory at Dwingeloo, an institution financed by the Netherlands Organization for Pure Research (Z.W.O.). The instrument has a beamwidth of 0.°56 at 21 cm. The great majority of the measures were made with a bandwidth of 20 kc/s. The total observing time was about 2,400 hours. That we could reach the accuracy required at the low intensities involved was entirely due to the efforts of Professor C. A. Muller, who designed the receiver and continually improved its stability.

* One of four papers presented in a symposium on radio astronomy at the Autumn Meeting of the National Academy of Sciences at the University of Indiana, November 17, 1959. Another paper from the symposium, by R. Minkowski, appears on pages 13–19. The remaining papers will be published as they are received.

† parsec (pc) = 3.26 light-years or 3.08×10^{18} cm.

‡ This temperature, the so-called "brightness temperature," gives the temperature of a black body which at the frequency concerned would give the observed surface intensity of radiation.

§ The longitudes are counted in the new system of galactic co-ordinates, from the longitude of the center as zeropoint; they have been indicated by the symbol l^{II}. For the sake of convenience we have used negative numbers to indicate longitudes below 360°.

‖ These observations extend in a somewhat less complete form beyond the plot shown in Figure 6; the above statement probably applies to the entire interval from −10° to +20° longitude.

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*INTERNATIONAL COOPERATIVE EFFORTS DIRECTED TOWARD OPTICAL IDENTIFICATION OF RADIO SOURCES**

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Radio positions of high accuracy permit us to consider as significant the presence of a faint optical object within the area given by the errors of the source position. But, positions of radio sources are not accurate enough to point uniquely to an optical object with which the source is to be identified. Supplementary evidence

is needed to establish an identification, such as the nature and the appearance of the optical object, its spectrum, the size of the source, and its radio spectrum. For distant objects and faint sources it can be difficult or even impossible to obtain the needed information. In that case, it is impossible to establish definitely a suspected identification.

Some sources could be identified with galactic and with extragalactic objects soon after the existence of radio sources had been discovered. Positions of relatively moderate accuracy pointed clearly to optical objects that were long known as highly peculiar. But the two strongest sources in the sky escaped identification until positions of high accuracy had been determined; both turned out to be faint and inconspicuous objects. This implied clearly that not all sources can be expected to be optically observable. But the initial success led to the expectation that it should be possible to identify many sources. Actually, as more sources became known very few additional identifications could be made. But, low accuracy of the positions of the fainter sources, of which many were later found to be spurious. could be considered as a possible explanation of the difficulty.

The surveys at 86.5 Mc/sec by Mills, Slee, and Hill[1] in Sydney, and at 159 Mc/sec (3C survey) by Ryle's group[2] in Cambridge, England, now provide positions that are accurate enough to justify a systematic search for identifications. Where the two surveys overlap, the agreement is excellent, although not complete. The accuracy of the positions of the Sydney survey permits to consider as possibly significant single galaxies brighter than photographic magnitude 16, and double galaxies of the types that are being considered as possible identifications brighter than photographic magnitude 19; somewhat fainter limits apply to the Cambridge survey. Support by the Science Foundation has permitted a systematic attempt to identify these sources on the plates of the National Geographic Society–Palomar Observatory Sky survey. During an extended visit of the author in Sydney, the 132 sources of the Sydney survey in the area from 0^h00^m to 6^h12^m, $-9°$ to $+3°$ were examined; in addition, this area was inspected for its content of peculiar galaxies. Mills has examined all sources between declination $-20°$ and $+10°$ that are: (*a*) more than $12^1/_2°$ distant from the galactic plane, with flux densities larger than 10^{-25} MKS units (230 sources); (*b*) identified in the Cambridge catalog (20 sources); (*c*) in the areas from 9^h24^m to 11^h24^m, $-21°$ to $+3°$, and from 23^h00^m to 23^h48^m. $-21°$ to $+3°$ (150 sources). The examination of the 480 sources that were examined has led to 59 objects that might provide identifications; a number of objects noted earlier are included. Dewhirst[3] has examined the 430 sources of the Cambridge catalog, which covers the sky north of $-10°$, and noted 14 objects. The results show that, as is to be expected, the three investigators have differed somewhat in their judgment which objects can be considered as providing possible identifications. The over-all results show definitely that for only a small fraction of the sources, of the order of 10 per cent, can objects be found that may provide an identification. The total of true identifications will be even smaller since a certain fraction of these objects should coincide only accidentally with a source. All objects noted are now under investigation with the 200-inch telescope. Whatever the final result will be, the conclusion is now unavoidable that with positions of the present accuracy not more than a small fraction of all sources can be identified. The implications of this result differ for various types of sources.

Emission nebulae are a type of source whose identification presents little difficulty. Both optical emission and radio emission of the ionized gas are due to the same physical process; the surface brightness is determined by the thickness of the ionized gas, the electron density, and the electron temperature. Thus optical and radio brightness are related, and the size of source and object must be identical. Moreover, the radio spectrum is typical; at very low frequencies the intensity increases with the frequency and becomes almost constant at high frequencies. The radio spectrum permits us to identify thermal sources as emission nebulae even if heavy obscuration hides the object. Very few sources of this type are in the surveys carried out at low frequencies. Since they are not important for the problems of identification, they have not been included in the data discussed in this paper.

In all other sources synchrotron radiation is the source of the radio emission. This non-thermal process does not necessarily contribute to the optical brightness. Intensity and size of the source thus are not related to brightness and size of the optical object. The radio spectrum shows a steep decrease of the intensity with increasing frequency. Except for a few sources that show a distinctly smaller gradient, the spectra of all sources seem to be quite similar. At the present stage, the radio spectrum does not contribute information that permits more than to separate thermal and non-thermal sources.

The sources may be divided into two classes.[4] Class I contains strong extended sources that are concentrated in the galactic plane. This class must contain objects in our Galaxy. Ten sources of this kind have now been identified.[5] Three of them are remnants of supernovae of type I: the supernova of 1054 (the Crab Nebula), Kepler's nova of 1604, and Tycho's nova of 1572, whose remnant was found on the basis of the position in the 3C survey. The other objects are the nebulosity of the Cassiopeia A source, probably a remnant of a supernova of type II that occurred 256 years ago, and a number of large nebulosities with diameters of several degrees. The Cygnus loop is the only object of the last group that was studied in detail. The results support the suggestion by Oort[6] that the Cygnus loop is a remnant of a supernova whose original fast expansion has been slowed down by interaction with the interstellar medium. This supernova must have been of type II. The age of the Cygnus loop can be estimated to about 50,000 years. It seems probable that the other objects are of similar origin.

It is possible to estimate very roughly the total number of such objects in the Galaxy. According to new unpublished results by Baade, the frequency of supernovae of type I and of type II is nearly equal—one supernova about every 400 year. That more remnants of supernovae of type II have been identified than of type I seems reasonable since supernovae of type II eject a mass of the order of several solar masses—much more than the supernovae of type I, whose remnants contain masses of the order of a tenth of a solar mass. The life of a remnant of a supernova of type II, therefore, can be much longer than that of a supernova of type I. The age of the Cygnus loop suggests a lifetime of the order of 50,000 years, which would lead to the expectation that the total number of such objects in the galaxy is of the order of 125. This is consistent with the possible number of galactic sources. That only 10 sources have been identified is easily understood as a consequence of the concentration to the galactic plane where obscuring interstellar dust clouds keep many objects from our sight.

Most sources are uniformly distributed over the sky and therefore are most likely external galaxies, although some extended sources might be features of the radio halo of the Galaxy. Normal galaxies are at most very weak emitters. Very few sources in the present surveys, therefore, are nearby bright galaxies. Only two single galaxies are definitely identified as strong sources. One, NGC 1316, does not appear to be very peculiar.[7] The other, NGC 4486 (M 87), has an outstanding peculiarity that was long known—a jet-like feature emerging from the nucleus. Peculiar as this feature is, it is not conspicuous enough to expect it to be visible in more distant galaxies, nor is it certain that it should occur in all galaxies in which the unknown process operates that causes the strong radio emission in NGC 4486. The fact that a galaxy coinciding with a source appears normal thus does not contradict an identification, which may have to remain tentative for lack of supporting evidence.

The second strongest source, Cygnus A, has been identified as a pair of colliding galaxies.[8] This identification, established on the basis of a precise position and supported by the appearance of the object and by its outstandingly peculiar spectrum, seemed to be supported by observations of the nebular redshift of a 21-cm absorption line. It is now established, however, that this absorption line does not exist. This does not contradict the identification. The fact that size and structure of the source differ from that of the object[9] may actually support rather than contradict the identification in the light of observations to be discussed below. NGC 1275, a double galaxy identified with a source, represents a well established example of a collision of galaxies.[10] Spectroscopic observations show that the object consists of two galaxies whose radial velocities differ by 3,000 km/sec; the two galaxies must be separated in a few million years. If collisions of galaxies lead to strong radio emission, the search for identifications may lead to the discovery of colliding galaxies even if such objects are rare. It is not sound, however, to assume that all double galaxies are due to collisions, nor does it seem permissible to think that all sources identified with double galaxies are due to collisions.

At the present stage of the investigation, it is not yet possible to state definitely how many of the coincidences noted in the present investigations will lead to definite identifications. But it is now quite definite that, as was suspected earlier,[11] some double and multiple elliptical galaxies are sources. Figure 1 shows some samples of new objects that seem to be definite identifications. Since single elliptical galaxies seem to be weaker emitters than spirals, and since the density of the gas in ellipticals is low, it is puzzling that identifications with double elliptical galaxies seem to be more frequent than those with doubles containing spirals.

The main reason why so few sources can be identified is probably to be sought in the fact that sources of the Cygnus A type are observable at distances where the galaxies are too faint to permit identification with positions of the present accuracy. Sources of this intrinsic strength should actually still be observable at distances where the optical object is beyond the reach of the 200-inch telescope. This interpretation is supported by an analysis of the available radio evidence by Ryle.[12] Unpublished results obtained at Jodrell Bank show that 10 to 15 per cent of the sources have angular diameters of less than 8″ and must indeed be more distant than Cygnus A, but of comparable intrinsic strength. The possibility cannot be excluded, however, that some sources might be truly invisible objects of an unknown

kind, such as features of the galactic halo or masses of gas in intergalactic space.

Observations of the size of sources often seem to contradict rather than to support identifications. A discordance between radio and optical size is understandable,

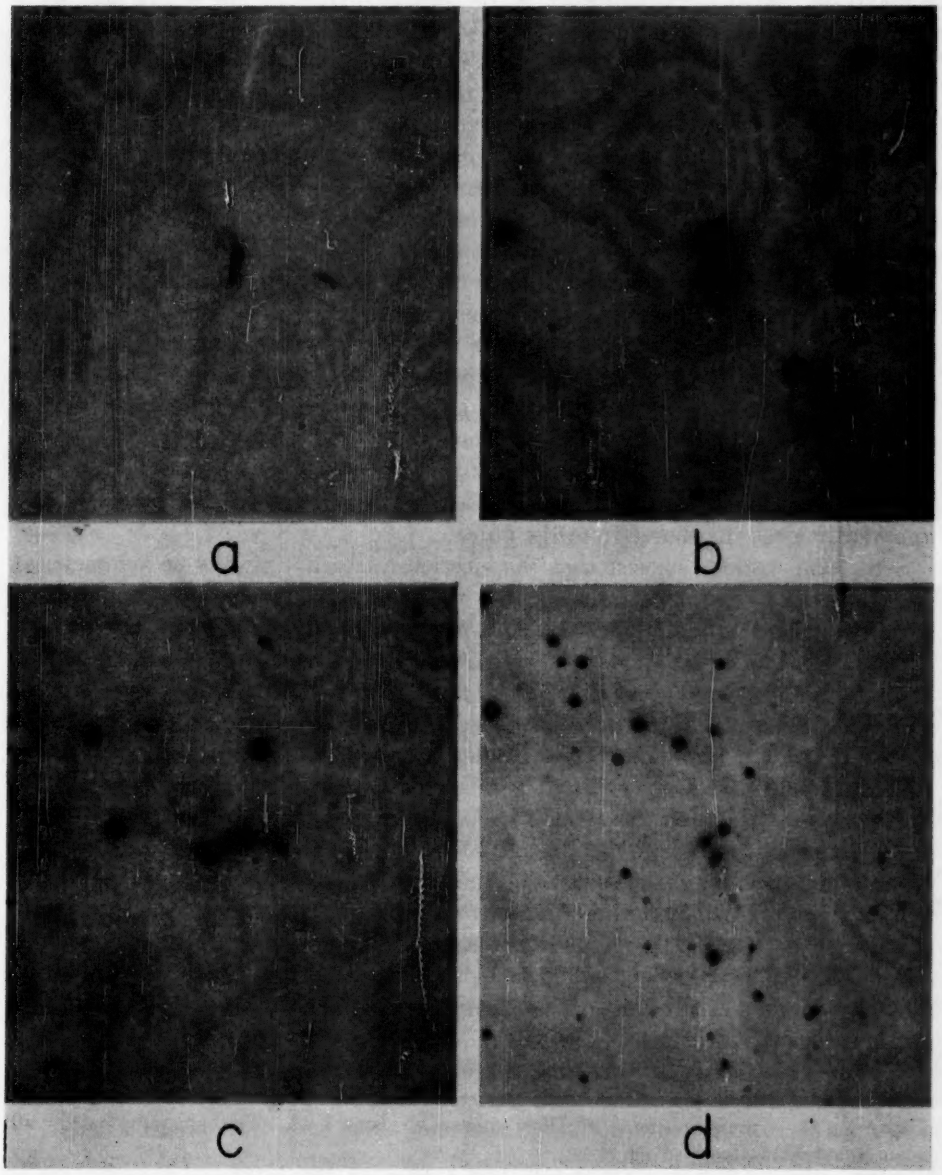

FIG. 1.—(*a*) Double galaxy, Sb + S0. Source Mills 00-015; 3C 26A.
(*b*) Double galaxy, E0 + E0. Source Mills 02 + 010; 3C 75A.
(*c*) Double galaxy, E1 + E0. Source 3C 310 *A.
(*d*) Double galaxy, E0 + Sb. Source 3C 433 *A.

since almost all the light of galaxies comes from their stars with a small contribution from emission nebulae, while the radio emission originates in tenuous gas from the motion of relativistic electrons in magnetic fields. Even normal galaxies are sur-

rounded by radio halos two or three times larger than the visible gas. But in some sources the difference between the optical appearance and the structure of the source is much greater. Cygnus A is identified with an object whose major axis is about 30″, or, at the distance 2.2×10^8 psc., 30,000 psc. The source appears as two masses, whose centers are separated by 82″ or 90,000 psc. and whose total extent is 134″ or 150,000 psc. A central source is not observed and must be fainter than about 10 per cent. That this difference of optical and radio appearance does not contradict the identification has now become strikingly clear from an unpublished investigation by Bolten of the source Centaurus A, whose identification with NGC 5128 cannot be doubted. In this case, the optical object has a major axis of 31′; the distance is not well established, but probably about 6×10^6 psc. The source contains a small central source that contains 25 per cent of the intensity. The outer parts of the source consist, except for a weak asymmetrical feature, of two symmetrical masses whose centers of gravity are separated by about 4°, or 400,000 psc. The total extent is almost 10°, or 1,000,000 psc. Except for the central source that seems stronger here than in Cygnus A, the structure of the two sources is similar, but in Centaurus A the dimensions of the source exceed the optical dimensions even more than in Cygnus A. The size of Centaurus A presents a serious problem. High velocities are necessary to transport the gas and the magnetic field to large distances. It is not clear at this time what are the processes that apparently eject the source from the object.

Some observations suggest that the ejection might not always be symmetrical. It seems now definitely established that the Hercules A source does not coincide with any galaxy brighter than the 20th magnitude. The diameter of the source is 2.′3. About 4′ south of the source is a peculiar double galaxy of about the 18th magnitude at a distance of 3.3×10^8 psc. Three interpretations are possible. The source might be an invisible gas mass, or it might be connected with a galaxy of 20th magnitude or fainter, thus even more extended than Centaurus A and intrinsically stronger than Cygnus A. The most conservative interpretation is that the source is connected with the peculiar galaxy; its distance from the galaxy would be 400,000 psc., comparable to the dimensions found in Centaurus A. The assumption of unilateral ejection of the source does not appear to be objectionable. Actually, at this time four other sources seem to present a similar but less well established situation. Such a dislocation of a source might make its identification difficult or impossible, but it seems probable that with positions of the present accuracy this difficulty does not arise often and is not more than a minor factor contributing to the difficulties of the identification problem.

* One of four papers presented in a symposium on radio astronomy at the Autumn Meeting of the National Academy of Sciences at the University of Indiana, November 17, 1959. Another paper from the symposium, by J. H. Oort, appears on pages 1–13. The remaining papers will be published as they are received.

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*ON THE MECHANISM OF CHLOROPHYLL-CYTOCHROME INTERACTION: THE TEMPERATURE INSENSITIVITY OF LIGHT-INDUCED CYTOCHROME OXIDATION IN CHROMATIUM**

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Most of the reactions in biological systems that have been studied in detail are thermal reactions in which collisions of molecules are necessary for activation. This is particularly true in enzyme-substrate interactions.[1] However, the increasing evidence for an ordered structure of biological systems, particularly in chloroplasts and mitochondria, has led to the consideration of alternative mechanisms,[2] particularly those which would allow energy transfer between stationary protein molecules.[3]

In the cytochrome chain, electron transfer can so far be explained by a restricted collision hypothesis.[4] On the other hand, the intermolecular energy transfer from carotenoid to chlorophyll appears to involve inductive resonance processes.[5, 6] An intramolecular energy transfer occurs in aromatic amino acid-heme systems[7] where light absorbed by the aromatic amino acids leads to a splitting of CO from heme.[8, 9] In this paper, we describe a light-induced intermolecular oxidation-reduction reaction between bacterial chlorophyll and a cytochrome of type c that is temperature independent (to 80°K) and that appears to require serious consideration of mechanisms for non-thermal oxidation-reduction reactions in living cells of this type.

Light-induced cytochrome oxidation in Chromatium has been shown[10] to involve a number of cytochromes, as is true of *R. rubrum*.[11, 12] A quantum requirement of 2 per electron[13] is found for the oxidation of a cytochrome of type c (cytochrome 423.5) by low intensity light absorbed by bacteriochlorophyll.

$$h\nu \longrightarrow \text{chl} \longrightarrow \text{cyto} \qquad (1)$$

Methods and Materials.—Kinetic measurements were made in the double-beam spectrophotometer[14] which has been adapted for recording absorbancy changes as

a function of time at liquid nitrogen temperatures. Identification of the component oxidized at low temperatures was made by the split-beam spectrophotometer[14] which records absorbancy changes as a function of wavelength. Both instruments provide infrared illumination of the suspension from a tungsten source filtered through a Wratten 88A filter. The tungsten source is used at about $^1/_3$ rated voltage and under these conditions gives adequately rapid cytochrome kinetics. The kinetics of oxidation of cytochrome suggest that the infrared intensities used caused effects equivalent to the Na line of about 10^{-3} milli Einsteins/liter/sec.[10] This corresponds to 10^{-10} Einsteins/sec. for the 0.1 ml volume. Chromatium was grown as described elsewhere[10] and was concentrated by centrifugation.

Results.—Figure 1 shows the spectroscopic response of the bacterial suspension

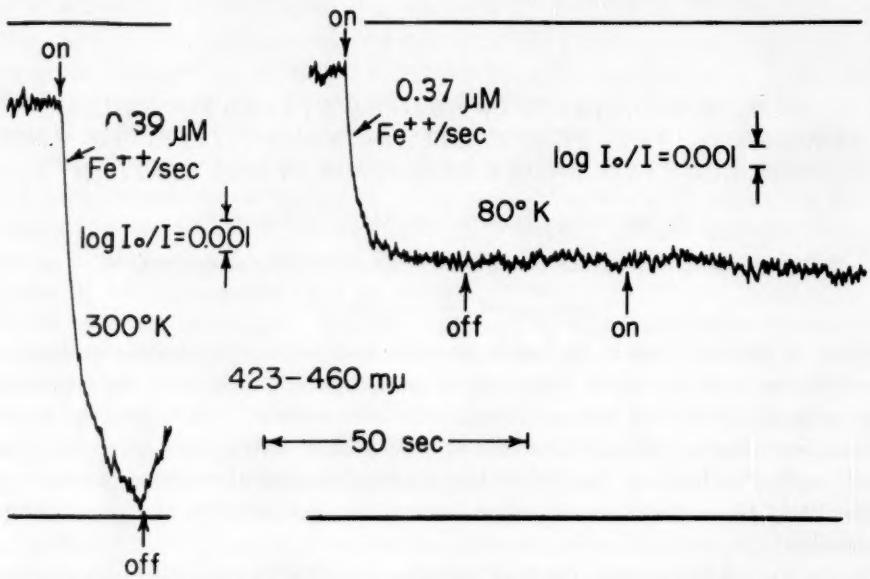

FIG. 1.—A comparison of the kinetics of cytochrome oxidation caused by illumination of an anaerobic suspension of intact cells of the photosynthetic bacterium Chromatium, at 300°K and at 77°K. The time and absorbancy scales are indicated on the figure. The infrared light had an intensity equivalent to an Na arc of roughly 10^{-3} milli Einsteins/liter/sec. The rates of oxidation of the cytochromes are calculated to be 0.39(left) and 0.37μM Fe^{++}/sec. on the assumption that the pigment has an extinction coefficient of approximately 100 cm^{-1} mM^{-1} at the two wavelengths indicated and at the two temperatures involved (optical path length 1 mm). (Expt. 11a).

to infrared illumination at 300°K (left). An abrupt downward deflection of the trace occurred at the moment of illumination, as indicated by the arrow, corresponding to a decrease of absorbancy at 423 mμ, as measured with respect to 460 mμ, and an oxidation of reduced cytochrome. A cessation of illumination after the recording was terminated caused a dark reduction of the cytochrome (not completely shown). In a repetition of the experiment at liquid nitrogen temperatures (~80°K) (the solution having been previously shielded from the measuring light until a few seconds before the infrared illumination was begun), infrared illumination again caused an abrupt downward deflection of the trace. In this case no reduction was observed on turning off the light for one hour (not shown). It is apparent from a comparison of the traces at 300°K and at 80°K (i) that the reaction proceeds to a substantial

extent at the lower temperature, (ii) that the half-time is not increased at the lower temperature, (iii) that the initial rate of the reaction, calculated as described in the figure, is no slower at the lower temperature, (iv) that the quantum requirement of the reaction is essentially the same at the lower temperature.

In order to identify the substance which is affected in the oxidation-reduction reaction at low temperatures and to show that it was converted from a ferrous to a ferric form, it is necessary to demonstrate the difference spectrum of the absorbancy changes at low temperature. For this purpose, the wavelength scanning split-beam spectrophotometer has been used, and a result for the visible region is shown in Figure 2. In this case, the absorbancy changes which occur on infrared illumination at room temperature are indicated by the open circles. It is seen that an absorption band having a maximum at ~553 mμ disappears upon illumination.[10] At the reduced temperature, the trace following the solid circles indicates the disappearance of an absorption band having an α peak at approximately 550 mμ with a corresponding β band at approximately 524 mμ. It is of considerable significance

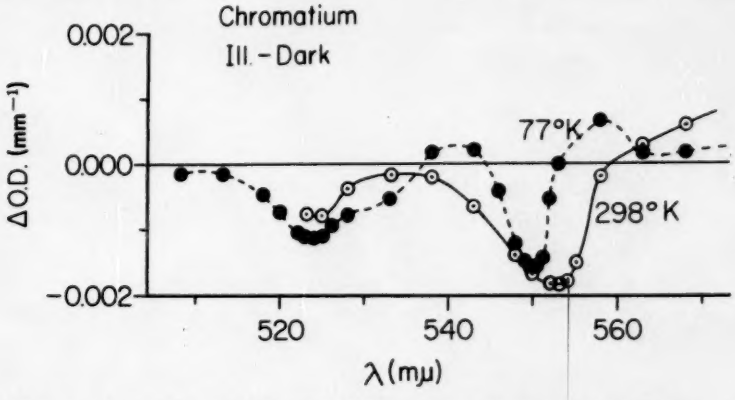

FIG. 2.—A recording of the difference spectrum between illuminated and dark Chromatium at 298°K and at 77°K. The experimental conditions are similar to those described in Fig. 1 (optical path length, 1 mm). (Expt. 21d).

that the absorbancy change shows only one α and one β peak. The low temperature spectra show a single Soret band at about 420 mμ. These results identify a single cytochrome involved in the light reaction at low temperature.

There is a high probability that an electron spin resonance signal accompanies this light-induced oxidation of a reduced cytochrome. Calvin[15] has obtained such a signal at low temperatures from the related bacterium *Rhodospirillum rubrum*. We have observed a room temperature signal from Chromatium but have not verified its existence at 80°K.[16] It is unlikely that such a signal is associated with the cytochrome[17, 18] and more likely that it is associated with an unpaired spin in chlorophyll.[15]

Discussion.—On the basis of these preliminary data, we conclude that infrared illumination of chlorophyll in the intact Chromatium cell initiates a temperature-insensitive electron or proton transfer reaction between bacterial chlorophyll and a closely associated cytochrome of type c (423.5 mμ). In this reaction, the cytochrome is oxidized from the ferrous to the ferric state with an over-all quantum requirement of about 2 per electron.[10] The reaction is not measurably slowed

from 300°K to 80°K, although the possibility that the reaction may be sensitive to temperatures below 80°K has not yet been eliminated.

The failure of this reaction to slow between 300°K and 80°K suggests that a special reaction mechanism between chlorophyll and cytochrome should be considered. For some time before these data were available, there had been considerable speculation on various reaction mechanisms; a suggestion based partially on inductive resonance was put forward by Kamen in 1957[19] but even then it appeared unlikely that an inductive resonance phenomenon between chlorophyll and cytochrome was involved in view of the very small absorbancy of the reduced cytochrome in the infrared region of the spectrum. Current thinking now tends towards proton migration[20] or charge transfer mechanisms. Explanations along these lines are described by Kamen[21] and Hill.[22] Calvin[15] has described semi-conductor mechanisms. These data at low temperatures now require a serious consideration of such possibilities.

The illumination of chromatophore of Chromatium would result in the absorption of photons in bacterial chlorophyll. If there were no cytochrome associated with the chlorophyll, there could follow an inductive resonance energy transfer to a particular chlorophyll molecule having cytochrome associated with it. The concentration of such chlorophyll-cytochrome complexes is not known, but a rough calculation, based on the magnitude of the observed absorbancy change at room temperature and the total chlorophyll absorption using ϵ values of about 100 cm^{-1} mM^{-1}, suggests that about 3 per cent of the chlorophyll could be so bound. The chlorophyll could then be transformed to an excited state and would accept an electron or a proton from the cytochrome, effectively forming an oxidized cytochrome and a reduced chlorophyll intermediate. Experiments on the detection of such a chlorophyll intermediate are currently in progress. Alternatively, an exciton would be transferred from chlorophyll to cytochrome. The mechanism of the reaction is not known, but the quenching of chlorophyll fluorescence by paramagnetic ions and the charge transfer mechanism proposed by Linschitz[23] are surely to be considered. Although the particular mechanism described here may not be appropriate, our attention is called for the first time in a biological system to experimental evidence for a temperature-independent intermolecular oxidation-reduction reaction.

The number of electrons transferred from cytochrome to chlorophyll would appear to be one electron per two quanta. This conclusion is based on the resemblance of the absorbancy changes to those for the one-electron oxidation of ferrocytochrome c to ferricytochrome c. Since the former has dominant absorption bands, our spectra identify the disappearance of this form but do not positively identify the oxidation state of the product. If a hypothetical higher oxidation state of cytochrome c is formed which has the same spectrum as the ferric state, our method would not detect it, and more than one electron could have been transferred per two quanta (two quanta/electron represents only 13 per cent efficiency[24]). The fact that an isolated cytochrome, probably identical to that in Chromatium, appears to have three heme groups[25] would make such a higher oxidation state rather unstable because of the possibility of electron transfers between the hemes.

In summary, our experiments do not eliminate the possibility that cytochrome is oxidized beyond the ferric state provided that such a state does not differ spectro-

scopically from the ferric state. However, the presence of other hemes in the cytochrome c molecule would render such a higher oxidation state unstable with the result that a net ferrous-ferric oxidation reaction would be observed.

In our studies of light-induced optical changes in "green" and "purple" photosynthetic systems,[12, 26, 27] the evidence for the formation of oxidizing equivalents at the level of cytochrome has always been consistent, but evidence for the formation of reducing equivalents has been equivocal.[26] Following Duysens'[28] and Olson's[29] work, however, we have found a significant discrepancy in the time relations of the formation of oxidizing and reducing equivalents in Chromatium at 300°K. This supports the idea presented here that the separation of oxidizing and reducing equivalents occurs between cytochrome and chlorophyll (i.e., oxidizes the former and reduces the latter) instead of between chlorophyll and pyridine nucleotide, and explains how the oxidizing equivalents may regularly be observed in cytochrome and irregularly at the level of pyridine nucleotide.

Others have claimed to be studying primary events in photosynthesis because of the rapidity with which they occur following intense flashes of light.[30] We present another criterion for a "primary" reaction: if the reaction occurs at a high quantum efficiency and with no change of rate at extremely low temperature, it may be a "primary" event. The reaction for Chromatium, described here, satisfies this criterion.

The generality of this reaction in Chromatium is not yet known, and the chlorophyll cytochrome interaction may differ in various types of cells. The ability to detect an infrared light-induced oxidation of cytochrome depends upon the dark cells containing some reduced cytochrome at low temperatures. This in turn depends upon the relative rates of decrease of the dark oxidase and reductase activities with temperature, which may differ in *R. rubrum* and Chromatium. We have not yet been able to detect this reaction in *R. rubrum* at 80°K. However, *R. rubrum* gives a free radical signal at low temperatures; it is therefore more likely that such a signal is associated with the acceptance of reducing equivalents by chlorophyll and not with cytochrome oxidation. Alternatively, the free radical signal may not correspond to a chlorophyll reaction at all, but to some unknown process; the electron spin resonance signal is very unspecific and does not identify the molecule involved as does the optical method.

The temperature-independent chlorophyll-cytochrome reaction observed in Chromatium is also of interest with respect to energy transfer between cytochromes. Whereas at room temperature several cytochromes are known to be affected by infrared illumination,[10] at liquid nitrogen temperatures no evidence for the participation of additional cytochromes is obtained, even with long intervals of illumination at high intensities. It must, therefore, be concluded that, whereas the electron transfer reaction between chlorophyll and one cytochrome may be non-thermal, that between cytochromes involves temperature-sensitive diffusion processes, as postulated for the operation of the carriers of the respiratory chain of mitochondria.[3]

These results, considered in the light of current interest in life at low temperatures,[31] indicate that a portion of the photosynthetic process in the immediate vicinity of chlorophyll occurs under very adverse environmental conditions. Investigations of the extent of formation of reduced substances such as pyridine nucleotide at these temperatures will be of interest.

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*STUDIES ON THE BIOSYNTHESIS OF PRODIGIOSIN IN SERRATIA MARCESCENS**

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Prodigiosin, the red pigment of *Serratia marcescens*, has been characterized as a tripyrrylmethene[1] (Fig. 1) although this structure has never been confirmed by synthesis. Its biosynthesis is of considerable interest both for its own sake and in relation to porphyrin biosynthesis where a tripyrrylmethane has been postulated as an intermediate by Turner[2] and, more recently, by Bogorad and Granick[3] and by Shemin.[4] In 1949 Hubbard and Rimington[5] investigated the biosynthesis of prodigiosin and showed that the nitrogen and the methylene carbon atoms of glycine are utilized in its biosynthesis whereas the carboxyl carbon atom is not; they also found that both carbon atoms of acetate are incorporated into the pigment. Since similar observations had been made in studies of heme biosynthesis Hubbard and Rimington concluded that prodigiosin biosynthesis should be given due consideration in the elaboration of any final scheme of porphyrin biogenesis.

During the last decade many of the details of porphyrin biosynthesis have been elucidated. It is now known that succinyl-Co A condenses with glycine to give

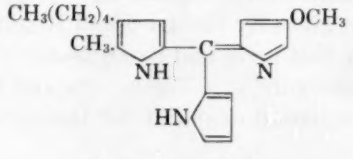

FIG. 1.—Prodigiosin.

5-aminolevulinic acid with the loss of CO_2 from the carboxyl group of glycine.[6] Two molecules of 5-aminolevulinic acid condense to form porphobilinogen which is then converted to uroporphyrinogen III. It was of interest to ascertain whether prodigiosin is indeed derived from 5-aminolevulinic acid and porphobilinogen as suggested by the data of Hubbard and Rimington. Another possibility which we felt merited consideration was that prodigiosin is derived from proline. This idea was put forward by Kost[7] on the basis of a study of the effects of amino acids on growth and pigment production of *Serratia marcescens*. The aim of the present investigation was to decide between these two alternatives.

Experimental.—5-Aminolevulinic acid hydrochloride-5-C^{14}, prepared from glycine-2-C^{14} by an unpublished method, was provided by Drs. F. Sparatore and D. Mauzerall of the Rockefeller Institute. Glycine-2-C^{14} was purchased from the Volk Radiochemical Company and L-proline-C^{14} from the Nuclear Chicago Corporation. We are grateful to Dr. James Moulder of the Department of Microbiology, University of Chicago, for a culture of *Serratia marcescens*.

In the experiments with 5-aminolevulinic acid-5-C^{14} and with glycine-2-C^{14} *Serratia marcescens* was grown on the medium of Williams *et al.*[8] For the experiments with L-proline-C^{14} the same medium was used except that a synthetic amino acid mixture was substituted for the enzymatic casein hydrolysate. This mixture

corresponded in composition to casein[9] except that hydroxyglutamic acid was omitted. The medium (250 ml. in a 5 l. diphtheria toxin bottle) was autoclaved and a solution of the C^{14} compound to be tested was sterilized by filtration and added just prior to the solidification of the agar. The inoculated flask was kept at room temperature in the dark and harvested after 5 days.

Prodigiosin was extracted from the bacteria by the alkaline extraction procedure described by Hubbard and Rimington[5] and then purified essentially as described by these authors. After extracting the pigment from petroleum ether (b.p. 30–60°C) with 85 per cent ethanol containing 1 per cent acetic acid, the ethanolic solution was diluted with an equal volume of water. The aqueous-ethanolic solution was then treated with aqueous sodium hydroxide until the color changed to orange, whereupon the pigment was extracted with petroleum ether. This procedure was repeated several times until the specific activity of the prodigiosin remained constant. In cases where the prodigiosin was found to be radioactive its specific activity was determined again after paper chromatography by a modification of the method of Williams *et al.*[8] Descending paper chromatography was used rather than circular chromatography between glass plates. A small amount of blue pigment, suggested previously to be a dimer of prodigiosin,[10] remained at the origin but the bulk of the pigment which was pink in color moved close to the solvent front. This was eluted with chloroform and in each case its specific activity was found to be unchanged. The concentration of prodigiosin was determined spectrophotometrically in chloroform containing 1 per cent acetic acid using the molar extinction coefficient given by Hubbard and Rimington.[5] The pigment was then plated as an infinitely thin layer and its radioactivity measured in a windowless gas flow counter (efficiency 39%). The sample and background were counted long enough to bring the standard deviation for the corrected count down to ±5 per cent.

Results and Discussion.—The results of these experiments are summarized in Table 1.

TABLE 1

UTILIZATION OF C^{14}-LABELED COMPOUNDS BY *Serratia marcescens*

C^{14}-Labeled Compound	Amount, mg	Total Activity, Counts/Min	Specific Activity of Labeled Compound, Counts/Min/mg of C	Specific Activity of Prodigiosin, Counts/Min/mg of C
Glycine-2-C^{14}	52.5*	2.17×10^6	129,500	1,550
5-Aminolevulinic acid hydrochloride-5-C^{14}	2.62	0.9×10^6	956,000	0†
L-Proline-C^{14}	40	2.2×10^6	105,200	3,560

* 50 mg of glycine-2-C^{14} was added and it was calculated from the composition of casein[9] that 2.5 mg of glycine was present in the medium.

† The sample (36.7 μ grams) and background were counted for 60 minutes each. The net count rate was −0.5 cpm and the S.D. ±1 cpm. Similar results were obtained with additional samples.

In confirmation of the results of Hubbard and Rimington[5] glycine-2-C^{14} was incorporated into prodigiosin. 5-Aminolevulinic acid-5-C^{14}, however, was not incorporated. Thus it appears that prodigiosin is not derived biosynthetically from the same pyrrolic precursor as porphyrins, viz., porphobilinogen. The possibility does exist that the 5-aminolevulinic acid was unable to pass through the cell membrane thus accounting for the lack of incorporation. However, experiments dem-

onstrating radioactivity in other cellular components derived from 5-aminolevulinic acid, such as porphyrins, purines, serine or methionine,[4] would exclude such a possibility. Observations made so far indicate that 5-aminolevulinic acid is able to penetrate the cell membrane: (a) Cells washed ten times with saline remained highly radioactive. (b) After extraction of the washed cells with 5 per cent trichloroacetic acid and then with chloroform-methanol, the residue was extracted with 10 per cent trichloroacetic acid at 90°C; the supernatant containing nucleic acids[11] and the residue containing crude protein were both found to be radioactive.

Results obtained with L-proline-C^{14} are in accord with the observation that 5-aminolevulinic acid is not a precursor of prodigiosin. L-Proline is approximately $2^1/_2$ times as efficient a precursor of prodigiosin as glycine whereas in heme synthesis, in the rat, according to Shemin and Rittenberg,[12] glycine is a much more efficient precursor than proline. All the evidence therefore favors L-proline, or a closely related derivative, rather than 5-aminolevulinic acid, as an intermediate in prodigiosin biosynthesis. It is possible that (a) glycine and proline are utilized for the biosynthesis of different parts of the prodigiosin molecule or (b) that the

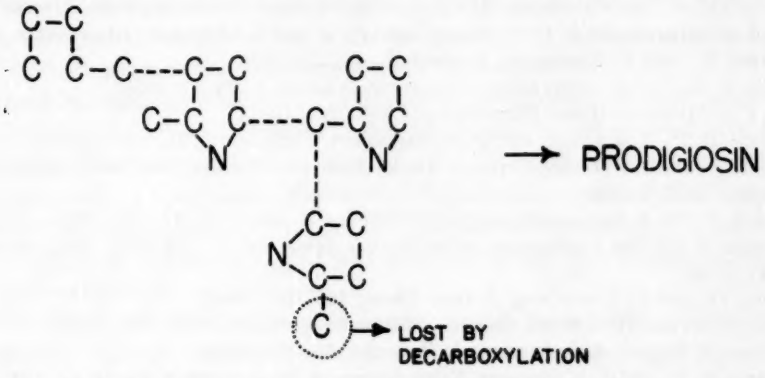

FIG. 2.—Hypothetical scheme for biosynthesis of prodigiosin from 5-carbon units.

methylene carbon and the nitrogen of glycine are incorporated into prodigiosin via proline or a closely related derivative. Experiments are now in progress to decide between these two possibilities. In any event, it is clear that the methylene carbon and the nitrogen of glycine are not incorporated into prodigiosin via 5-aminolevulinic acid. Furthermore, the pyrrolic precursor of prodigiosin found in a *Serratia marcescens* mutant,[13] having the formula $C_{10}H_{10}N_2O_2$, may also be derived from proline or a closely related derivative. Two hypotheses of prodigiosin biosynthesis,[14, 15] both of which are based on the assumption that prodigiosin and porphyrin biosynthesis are closely linked, appear to require reconsideration in the light of the present observations. It would seem rather that prodigiosin biosynthesis is closely linked with the metabolism of 5-carbon units, such as proline, ornithine, and glutamic acid, and that *Serratia marcescens* is an excellent organism for profitably studying the interrelationships of these acids.

As a working hypothesis we would like to suggest that the pyrrolic precursor from the *Serratia marcescens* mutant as well as prodigiosin are built up of 5-carbon units and that the amyl group in prodigiosin is derived from a similar deaminated 5-carbon unit. The proposed scheme as depicted in Figure 2 is based on the as-

sumption that the structure of prodigiosin is correctly represented in Figure 1. Furthermore, inspection of the prodigiosin molecule suggests that at least the methoxyl-bearing ring may be derived directly from hydroxy-proline.

Summary.—Glycine-2-C^{14} was incorporated into prodigiosin, the red pigment of *Serratia marcescens*, in confirmation of the results of Hubbard and Rimington. 5-Aminolevulinic acid-5-C^{14} was, however, not incorporated showing that the 3 pyrrole rings of prodigiosin must be derived from a precursor other than porphobilinogen, the pyrrolic precursor of porphyrins. Experiments with L-proline-C^{14} indicated that proline or a closely related metabolic derivative is a precursor of prodigiosin.

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*THE ROLE OF TRANSCARBOXYLATION IN PROPIONIC ACID FERMENTATION**

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The evidence that propionibacteria form propionate by the direct decarboxylation of succinyl CoA[1] has been reviewed by Wood *et al.*[2] It has generally been considered that carbohydrates, e.g., glucose, glycerol, etc. are catabolized to pyruvate which is then converted to oxalacetate by the fixation of carbon dioxide. Oxalacetate is reduced to succinate, which in turn is esterified with CoA and decarboxylated to propionyl CoA and CO_2. Therefore the reduction of one mole of pyruvate to propionate would be expected to involve the fixation and release of one mole of CO_2.

Because succinate is symmetrical, half of the CO_2 released would be derived from the original fixation, and the other half from pyruvate. However, in isotope experiments designed to measure these reactions, the observed turnover of CO_2 was insufficient to account for the propionate formed and it was concluded that a one-carbon unit other than free CO_2 was a product of the cleavage.[2, 3] Delwiche *et al.*[4] and Phares *et al.*[5] also obtained evidence suggesting that a one-carbon unit was formed during the decarboxylation of succinate; isotopic propionyl CoA was incorporated into succinyl CoA by extracts of *Propionibacterium pentosaceum* more rapidly than labeled CO_2. Further evidence for the formation of a C_1 compound other than CO_2 was obtained in studies of the randomization of the C^{14} of propionate-3-C^{14} to propionate-2,3-C^{14} by resting cell suspensions of *P. arabinosum*.[2] This conversion presumably occurred via a carboxylation yielding a catalytic amount of a symmetrical C_4 dicarboxylic acid followed by decarboxylation. On the other hand there was little fixation of CO_2 during this reaction, nor was there loss of C^{14} from the carboxyl group of $C^{14}H_3—CH_2—C^{14}OOH$ during the randomization of the C^{14} in carbons 2 and 3. However, Pomerantz,[6] by the mass analysis of the products formed from C^{13} triply-labeled propionate, was able to show that there is cleavage between carbons 1 and 2 yielding a C_1, presumably by formation of a C_4 dicarboxylic acid, randomization, and subsequent cleavage.

Succinate has also been shown to be involved in the catabolism of propionate in animal tissues. Lardy and Peanasky[7] and Lardy and Adler[8] showed with extracts of liver mitochondria that CO_2 is fixed in an ATP-dependent reaction with propionyl CoA yielding succinate. Flavin and Ochoa[9] demonstrated that the primary product of the carboxylation of propionyl CoA was not succinyl CoA but methylmalonyl CoA, and that succinyl CoA was only formed in a subsequent reaction from methylmalonyl CoA.

The elucidation of this pathway in animal tissues raised the question whether succinate was directly decarboxylated or whether methylmalonate was an intermediate in the formation of propionate by propionic acid bacteria. Therefore the present studies were designed to investigate the pathway of propionate formation in cell-free extracts of *P. shermanii* and to ascertain the fate of the one-carbon unit. *P. shermanii* was chosen because Wood, Kulka, and Edson[10] have shown that extracts from this culture fix CO_2, bring about the randomization of C^{14} in propionate formed from glucose-1-C^{14}, and remain active for several hours at 30°.

The results indicate that succinyl CoA is not decarboxylated but is first converted to methylmalonyl CoA and that it is the latter compound which is cleaved to propionyl CoA. This cleavage occurs with transfer of the carboxyl carbon to pyruvate, forming oxalacetate, and the C_1 unit does not equilibrate with free CO_2. Thus it is not CO_2 as such but a C_1 unit which combines with pyruvate to form oxalacetate, while the fixation of CO_2 *per se*, may occur with phosphenol pyruvate.[11, 12]

The results may be summarized by the following set of equations:

$$\text{Succinyl CoA} \rightleftarrows \text{Methylmalonyl CoA} \quad (1)$$

$$E^* + \text{Methylmalonyl CoA} \rightleftarrows \text{Propionyl CoA} + E—C_1 \quad (2a)$$

$$E—C_1 + \text{Pyruvate} \rightleftarrows \text{Oxalacetate} + E \quad (2b)$$

$$\text{Oxalacetate} + 4H \rightleftarrows \text{Succinate} \quad (3)$$

$$\text{Succinate} + \text{Propionyl CoA} \rightleftarrows \text{Succinyl CoA} + \text{Propionate} \quad (4)$$

$$\text{Net: Pyruvate} + 4H \rightleftarrows \text{Propionate} \quad (5)$$

* E = Enzyme.

Reactions 2a and b involve a transcarboxylation, which is a novel biochemical reaction.

Methods.—Propionibacterium shermanii (52W) was maintained in lactate-glucose stab cultures and grown for 3 days at 30° in 900 ml of glucose-yeast extract medium[13] in 1-liter flasks fitted with cotton plugs. The cells were harvested by centrifugation and washed with 10 volumes of distilled water. The yield was 4–5 gm (wet wt.) of cells/liter of medium and the cells were lyophilized and stored at −10°.

A cell-free extract was prepared with the aid of the Nossal shaker.[14] Each gram of dry cells was suspended in 16 ml of a solution of KCl (0.12 *M*) and L-cysteine (0.001 *M*) at pH 7, mixed with an equal volume of glass beads (Ballotini No. 13). The mixture was then shaken for 15 sec and recooled for 5 min; the process was repeated 4–6 times. The glass beads were removed by centrifugation at 13,000 × g for 15 min and the remainder of the cell debris by centrifugation at 35,000 × *g* for 30 min. The supernatant solution was passed through a column of Dowex 1-Cl^- (0.5 gm/ml of extract) to remove nucleotides.[15] The eluate was next brought to 90 per cent saturation with neutralized ammonium sulfate, centrifuged, and the precipitate was washed once with 20 volumes of 90 per cent ammonium sulfate solution. The precipitate was dissolved in 5 volumes of 0.1 *M* potassium phosphate buffer at pH 7 containing 0.001 *M* cysteine. This solution was dialyzed overnight against 20 volumes of the same buffer; after the first 3 hours the dialyzing fluid was replaced by fresh buffer All operations were carried out at 0–4° and the extract contained about 15–20 mg of protein per ml as measured by the biuret reaction.[16] The extracts were stored at −12° and remained active for a month or longer.

Sodium propionate-3-C^{14} and succinic acid-1,4-C^{14} were obtained from Volk Radiochemical Co. Propionic acid anhydride-C^{14} was prepared by the method of Orshansky and Bograchov.[17] Dry sodium propionate (1.1 gm) was thoroughly stirred with sulfur (0.055 g) dissolved in bromine (0.6 gm). The anhydride was distilled under reduced pressure and was 90 per cent pure. The yield was 70–80 per cent. Succinic acid anhydride-C^{14} was prepared by refluxing succinic acid-C^{14} with acetic anhydride. The acetic anhydride was removed by distillation and the last traces were removed by briefly washing with cold ether. Methylmalonic acid was synthesized from methyl iodide and sodium diethylmalonate[18] and isolated by partition chromatography on Celite.[19] The coenzyme A derivatives were prepared from the appropriate acid anhydrides by a modification[20] of the method of Simon and Shemin.[21] When only the acid was available, the mixed anhydride method described by Beck *et al.*[22] was employed.

Oxalacetate-4-C^{14} was prepared from oxalacetate by an exchange with $C^{14}O_2$ in the presence of Mn^{++}, ITP, and phosphoenol pyruvate carboxykinase.[23] Carrier oxalacetic acid was added and the product was isolated by elution from Celite with $CHCl_3$ containing 15 per cent of *n*-butanol.[19]

In order to measure the incorporation of propionyl-C^{14} CoA into the mixed dicarboxylic acids (succinate and methylmalonate), the appropriate compounds were incubated in potassium phosphate buffer at pH 7 at 30°. The reaction was stopped by the addition of 2 drops of 2 *N* NaOH, and the CoA derivatives were hydrolyzed for 5–10 min in a boiling water bath. The reaction mixture was made acid with 2 drops 6 *N* HCl, mixed with 2–3 gm of Celite and packed into a column 15 mm in diameter. The organic acids were eluted with 25 ml of ether and transferred into 1 ml of dilute alkali containing bromthymol blue by evaporating the ether. The alkaline solution was evaporated to dryness on planchets under an infrared lamp and the radioactivity was measured in a gas flow counter. The mixture was next made acid with dilute HCl and re-evaporated, whereupon propionic acid distilled off quantitatively leaving only the non-volatile, dicarboxylic acids to be counted.

When it was desired to isolate propionic, methylmalonic, and succinic acids individually, 5–10 μmoles of each acid was added as carrier to the alkaline reaction mixture and evaporated to dryness. The residue was dissolved in 0.5 ml of 0.5 *N* H_2SO_4, mixed with 1 gm of Celite, and packed into a 5-gm column of Celite prepared according to the procedure of Swim and Krampitz.[19] Propionic acid was eluted with 50 ml of $CHCl_3$, methylmalonic acid with 50 ml of $CHCl_3$ containing 7.5 per cent of *n*-butanol, and succinic acid with 50 ml of $CHCl_3$ containing 10 per cent of *n*-butanol. The compounds were located by titration, and the alkaline layer was removed and evaporated to dryness on planchets for measurement of the radioactivity.

Results.—Exchange of propionate-C^{14} with succinate: It has been observed with resting cells,[2, 3] and with extracts of propionibacteria[4, 5] that propionate is "equilibrated" with succinate. It is seen in Table 1 that the present extracts bring about a similar exchange; however, the conversion is believed to occur by reactions 1 and

TABLE 1

THE CARBOXYLATION OF PROPIONYL-C^{14} CoA BY SUCCINATE OR CO_2

Exp. No.	Substrate*	C^{14}-Dicarboxylic Acid Formed† μMoles
1	Propionyl-C^{14} CoA + succinyl CoA	0.084
2	Propionate-C^{14} + succinate + CoA + ATP	0.090
3	Propionate-C^{14} + succinate + ATP	0.002
4	Propionate-C^{14} + succinate + CoA	0.033
5	Propionate-C^{14} + $KHCO_3$ + ATP + CoA	0.030

* When indicated the system contained (in μmoles): propionate-C^{14} or propionyl-C^{14} CoA, 0.3; succinate or succinyl CoA, 1; $KHCO_3$, 50; ATP, 3; CoA, 1 and in addition potassium phosphate, pH 7, 50; enzyme with 4 mg of protein. Final volume, 0.5 ml.; incubated 30 min at 30°.

† Presumably a mixture of succinic and methylmalonic acids. The μmoles are calculated from the total non-volatile radioactivity and the specific activity of the propionate.

2a. In Exp. 1, Table 1, the incorporation of C^{14} is consistent with conversion of the succinyl CoA to methylmalonyl CoA (reaction 1) and exchange of propionyl-1-C^{14} CoA with the methylmalonyl CoA (reaction 2a). In Exp. 2 when CoA derivatives were not added, the derivatives were synthesized in the presence of CoA and ATP. Omission of CoA (Exp 3) or ATP (Exp 4) greatly decreased the reaction. Exp. 5 with CO_2, ATP, CoA, and propionate-1-C^{14} involves a net synthesis of dicarboxylic acids, presumably with conversion of the CO_2 to the active C_1. When extracts were prepared from lyophilized cells which had been stored for sev-

eral months, there was no activity with CO_2 but there was transfer of the C_1 from methylmalonyl CoA to pyruvate (reaction 2). This observation lends support to the postulate that the transcarboxylation reaction involves a C_1 unit which is not CO_2.

Formation of dicarboxylic acids from propionyl CoA and oxalacetate: Reaction 2a involves the formation of the C_1 unit from methylmalonyl CoA. It was of interest to learn whether other compounds which were known to yield Co_2 during their catabolism would also form a C_1 unit which would be transferred to propionyl CoA. It is seen in Table 2 that in the presence of succinate, ATP, and CoA there was con-

TABLE 2

THE CARBOXYLATION OF PROPIONYL-C^{14} CoA BY VARIOUS COMPOUNDS

Additions*	C^{14} in Dicarboxylic Acids Formed† μMoles
Succinate	0.036
Oxalacetate	0.117
Oxalacetate + malonate	0.120
Malate	0.016
Fumarate	0.006
Isocitrate	0.002
α-Ketoglutarate	0.003
$KHCO_3$‡	0.014

* The system contained (in μmoles): potassium phosphate, pH 7, 50; propionate-C^{14}, 0.3; CoA, 1; ATP, 1; enzyme with mg of protein; and where indicated, succinate, oxalacetate, isocitrate, α-ketoglutarate, malate, fumarate, 1; malonate, 3; $KHCO_3$, 50. Final volume, 0.5 ml; incubated 30 min at 30°.

† Calculated as described in footnote to Table 1.

‡ 3 μmoles of ATP were present in this case.

version of propionate-C^{14} to dicarboxylic acids, thus confirming Exp. 2, Table 1. However, oxalacetate was much more effective than succinate. Forty per cent of the propionate was converted to nonvolatile acids in the presence of oxalacetate and higher yields have been observed in subsequent trials. These results are in accord with reaction 2b in which a C_1 unit would be formed from oxalacetate and transferred to propionyl CoA to form methylmalonyl CoA, reaction 2a. Other experiments in Table 2 indicate that oxalacetate probably was not converted to succinate prior to transcarboxylation with propionyl CoA. The presence of 3 μmoles of malonate was not inhibitory. Also, several of the possible intermediates in the reduction of oxalacetate to succinate were tested and none was as active as oxalacetate itself. It is not likely that oxalacetate was first decarboxylated and the free CO_2 fixed with propionyl CoA since the yield of dicarboxylic acids was only about one-tenth as great with bicarbonate as with oxalacetate. Furthermore, other extracts, which were prepared from aged cells and which would not fix CO_2, readily utilized oxalacetate for the formation of the dicarboxylic acids from propionyl CoA. Formaldehyde, formate, pyruvate, and 6-phosphogluconate were also inactive in the transcarboxylation. It is not known, however, whether these compounds are metabolized by the extracts; therefore, the results may be negative because the required enzymes were not present to form the C_1 compound which is transferred to propionyl CoA.

The final products of the transcarboxylation reaction between propionyl CoA and oxalacetate are succinyl CoA and pyruvate (reactions 2b, 2a, and 1, right to left). Succinyl CoA was demonstrated by the preparation and chromatographic

separation of radioactive succinhydroxamic acid.[24] The pyruvate was determined by the oxidation of DPNH with lactic dehydrogenase (Table 3). Although there

TABLE 3

THE FORMATION OF PYRUVATE FROM OXALACETATE AND PROPIONYL CoA

Substrate*	Pyruvate Formed† μMoles
Oxalacetate + propionyl CoA	0.56
Oxalacetate	0.17

*The system contained (in μmoles): oxalacetate, 2; propionyl CoA, 1; sodium phosphate, pH 7, 50; and enzyme with 4 mg of protein. Final volume, 1 ml; incubated 10 min at 30°.

† Reaction was stopped by the addition of TCA to give a concentration of 2 per cent and the preparation was centrifuged and neutralized. Pyruvate was assayed in an aliquot by observing the change in O.D. at 340 mμ after the addition of 0.4 μmole of DPNH and lactic dehydrogenase.

was considerable decarboxylation of oxalacetate to pyruvate in the absence of propionyl CoA, the addition of propionyl CoA markedly increased the formation of pyruvate. No phosphoenol pyruvate was detected by enzymic assay with pyruvic kinase, ADP, DPNH, and lactic dehydrogenase.

The formation of oxalacetate from pyruvate and a C_1 unit (reaction 2b) was investigated using succinyl-1,4-C^{14} CoA as the C_1 donor, i.e., via methylmalonyl CoA. The oxalacetate formed was separated from the residual pyruvate by partition chromatography and degraded as follows.[25] The β-carboxyl group was released by treatment with 0.1 volume of 0.1 *M* $Al_2(SO_4)_3$ for 60 min. The resulting pyruvate was isolated by elution from Dowex 1-Cl^- with 0.02 *N* HCl and was decarboxylated with $Ce(SO_4)_2$. The CO_2 formed in each degradation was collected in alkali and plated as $BaCO_3$. All of the radioactivity in the oxalacetate carboxyl groups was present in the β-carboxyl moiety. Had oxalacetate arisen from succinate by oxidation, radioactivity would have been present in both carboxyl groups.

In addition to oxalacetate, propionyl CoA should be formed in the over-all reaction of succinyl CoA with pyruvate. There was some formation of propionyl-C^{14} CoA from succinyl-1,4-C^{14} CoA in the absence of pyruvate, but the addition of pyruvate increased the yield of propionyl C^{14} CoA almost two-fold. In addition to propionyl CoA and oxalacetate some $C^{14}O_2$ was produced and the amount was increased in the presence of pyruvate. The $C^{14}O_2$ presumably was formed by decarboxylation of oxalacetate. In the absence of added pyruvate the $C^{14}O_2$ may have arisen by conversion of the C_1 unit of reaction 2a to CO_2 or by transcarboxylation to trace amounts of pyruvate, reaction 2b, and subsequent enzymatic decarboxylation of oxalacetate. Phosphenol pyruvate was much less active than pyruvate in promoting the decarboxylation of succinyl CoA.

Demonstration of methylmalonyl CoA as a reactant: Flavin and Ochoa[9] showed in mammalian tissue preparations that methylmalonyl CoA is the primary product of the carboxylation of propionyl CoA by CO_2. This compound has now been shown to be the primary product of the transcarboxylation reaction in propionic acid bacteria (reactions 2a and 2b). Propionyl CoA and oxalacetate-4-C^{14} were incubated with the bacterial extract for various periods, the reaction was halted, and carrier methylmalonate and succinate were added to the reaction mixture and re-isolated chromatographically.

TABLE 4

RELATIVE YIELDS WITH TIME OF METHYLMALONATE AND SUCCINATE FORMED FROM PROPIONYL CoA AND OXALACETATE-4-C^{14}

Duration of Incubation min	Methylmalonate Formed μMoles*	Succinate Formed μMoles*	Total Acids Formed μMoles
1	0.139	0.032	0.171
5	0.170	0.071	0.241
20	0.125	0.125	0.250
40	0.058	0.247	0.305

* Calculated on the basis of the total radioactivity recovered and from the specific activity of the oxalacetate.

The complete system contained (in μmoles): potassium phosphate, pH 7, 50; propionyl CoA, 0.4; oxalacetate-4-C^{14}, 1; and enzyme with 4 mg. of protein. Final volume, 0.5 ml; incubated as indicated at 30°.

As shown in Table 4, both labeled methylmalonate and succinate were present, indicating that the CoA esters had been formed from oxalacetate-C^{14} and propionyl CoA. Maximum methylmalonate formation occurred within 5 min and thereafter decreased, while succinate formation, which initially lagged behind that of methylmalonate increased continuously. After 1 min methylmalonate constituted 82 per cent of the total dicarboxylic acid while at 45 min it was only 19 per cent of the total.

Further evidence that methylmalonyl CoA and not succinyl CoA is the reactant in the transcarboxylation was obtained by incubating extract and pyruvate and either methylmalonyl CoA or succinyl CoA for 1 min and measuring oxalacetate formation with malic dehydrogenase. Table 5 shows that more than 10 times as much

TABLE 5

FORMATION OF OXALACETATE FROM PYRUVATE AND METHYLMALONYL CoA OR SUCCINYL CoA

Substrate*	Oxalacetate Formed† μMoles
Pyruvate + methylmalonyl CoA (0.65 μmole)‡	0.325
Pyruvate + methylmalonyl CoA (0.4 μmole)‡	0.203
Pyruvate + succinyl CoA (0.58 μmole)	0.022
Pyruvate + succinyl CoA (0.58 μmole)	0.020

* System contained potassium phosphate, pH 7, 50 μmoles; pyruvate, 10 μmoles; methylmalonyl CoA or succinyl CoA as indicated and enzyme with 2 mg of protein. Final volume, 0.5 ml; incubated 1 min at 30°.

† Reaction was stopped by the addition of TCA to give a concentration of 2 per cent and the preparation was centrifuged and neutralized. Oxalacetate was assayed in an aliquot by observing the change in O.D. at 340 mμ after the addition of 0.4 μmole of DPNH and malic dehydrogenase.

‡ As active isomer.[26]

oxalacetate was formed from methylmalonyl CoA as from succinyl CoA during the brief reaction period. Therefore, it seems reasonable to conclude that the compound which is the immediate C_1 donor is methylmalonyl CoA rather than succinyl CoA. It also seems clear that the rate-limiting reaction is the conversion of succinyl CoA to methylmalonyl CoA, since upon longer incubation the yield of oxalacetate from succinyl CoA was similar to that obtained with methylmalonyl CoA.

The role of biotin: Delwiche[27] has shown that resting cells of *P. pentosaceum* grown on a medium low in biotin decarboxylate succinate slowly and that the addition of biotin stimulates this decarboxylation. Therefore experiments were performed to ascertain the effect of avidin, an inhibitor of biotin activity, on the exchange between succinate and propionyl CoA and on the transcarboxylation

TABLE 6

EFFECT OF AVIDIN AND BIOTIN ON THE CARBOXYLATION OF PROPIONYL-C^{14}CoA BY OXALACETATE OR SUCCINATE

Additions*	C^{14}-Dicarboxylic Acids Formed in the Presence of: Oxalacetate μMoles†	Succinate μMoles†
None	0.129	0.054
Avidin	0.002	0.002
Avidin + biotin	0.140	...
Biotin	0.122	...

* The system contained (in μmoles): potassium phosphate, pH 7, 50; propionyl-C^{14}CoA 0.3; oxalacetate or succinate, 1; enzyme containing 4 mg of protein; and, where indicated, biotin, 0.08, avidin, 200 μg. Final volume, 0.5 ml; incubated 30 min at 30°.
† Calculated as described in footnote to Table 1.

between oxalacetate and propionyl CoA in extracts of *P. shermanii*. It is seen in Table 6 that the addition of 200 μg of avidin (0.5 units of activity) almost completely inhibited the transfer of a carboxyl moiety from oxalacetate or from succinate (via methylmalonyl CoA). This inhibition was prevented by the addition of 0.08 μmole of biotin simultaneously with the avidin. The addition of biotin to the extract in the absence of avidin did not enhance the transcarboxylation reaction. A similar relationship between avidin and biotin has been reported by Wakil *et al.*[28] in their studies of the formation of malonyl CoA from acetyl CoA and CO_2. Recently, Lynen *et al.*[29] have presented evidence of the formation of a CO_2-biotin-enzyme complex as an intermediate in the fixation of CO_2 with β-methylcrotonyl CoA. The inhibition by avidin indicates that biotin also functions as a cofactor in the transcarboxylation reaction and it may be mediated by a complex similar to that formulated by Lynen *et al.*

The isomerization of methylmalonyl CoA and succinyl CoA (reaction 1): It has been suggested that the mechanism of conversion of methylmalonyl CoA to succinyl CoA is a transcarboxylation reaction, i.e., that the unesterified carboxyl group of methylmalonyl CoA is transferred to the β-carbon of propionyl CoA to form succinyl CoA.[26] If this were also the mechanism in the bacterial system, it seemed possible that both the formation of methylmalonyl CoA and its subsequent conversion to succinyl CoA might be inhibited by avidin. However, that was not the case. Table 7 summarizes the results of two experiments in which methyl-

TABLE 7

THE EFFECT OF AVIDIN ON THE FORMATION OF METHYLMALONATE AND SUCCINATE FROM PROPIONYL CoA AND OXALACETATE-C^{14}

Exp. No.	Conditions‡	Methylmalonate Formed* μMoles	Succinate Formed* μMoles
1	Complete system + avidin, 5 min	0.002	0.001
2a	Complete system, 5 min	0.155	0.104
2b	Complete system, 5 min,† fresh enzyme, 30 min	0.020	0.377
2c	Complete system, 5 min,† fresh enzyme + avidin, 30 min	0.014	0.246
3a	Complete system, 5 min	0.195	0.027
3b	Complete system, 5 min,† fresh enzyme, 30 min	0.019	0.320
3c	Complete system, 5 min,† fresh enzyme + avidin, 30 min	0.013	0.208

* Calculated as described in footnote to Table 4.
‡ The complete system contained (in μmoles): potassium phosphate, pH 7, 50; propionyl CoA, 0.5; oxalacetate-4-C^{14}, 1; enzyme with 4 mg of protein in Exp. 1 and 2, with 2 mg of protein in Exp. 3 and 200 μg of avidin where indicated.
† The incubation was stopped after 5 min by the addition of acid, the preparation was neutralized, and fresh enzyme was added with or without 200 μg of avidin. Incubation was then continued for 30 min at 30°.

malonyl CoA was synthesized from oxalacetate-4-C^{14} and propionyl CoA by a 5-min incubation with the extract. The reaction was stopped by the addition of acid. An aliquot of the preparation was fractionated to determine the amount of methylmalonyl CoA and succinyl CoA formed during the initial incubation. Two aliquots of the mixture were then neutralized and incubated with fresh extract, one portion of which had been pre-incubated with avidin for 5 min. The two reactions were then allowed to proceed for 30 min and the methylmalonate and succinate were isolated. It is seen that during the 30-min incubation, the methylmalonyl CoA was utilized in both the presence and absence of avidin. In the presence of avidin the increase in succinate was equivalent to the decrease of the methylmalonate. In the absence of avidin the amounts of succinate and of total dicarboxylic acids were greater than in the avidin-treated mixture because additional transcarboxylation from oxalacetate was occurring during the 30 min.

Flavin *et al.*[30] obtained complete inhibition of the conversion of methylmalonyl CoA to succinyl CoA by the addition of malonate. However, when extracts of propionibacteria were incubated with propionyl CoA and oxalacetate-C^{14}, the amount of succinate formed was the same with or without malonate. The possibility exists that the failure of malonate to inhibit the isomerization was due to the demonstrated inability of these extracts to convert malonate to malonyl CoA, a compound which would be more likely to inhibit the reaction.

Recently Smith and Monty[31] have reported that the methylmalonyl isomerase activity is considerably lower in vitamin B_{12}-depleted rats than in control rats. Barker *et al.*[32] have found that the conversion of glutamate to β-methylaspartate requires a heat stable coenzyme containing pseudovitamin B_{12}. The coenzyme could be removed from their enzyme preparation by treatment with charcoal. In view of the results of Smith and Monty it seemed possible that the isomerase might involve a B_{12} coenzyme; however, treatment of extracts of propionibacteria with charcoal failed to inhibit isomerization of methylmalonyl CoA.

Formation of CoA derivatives of succinate, propionate, and methylmalonate: Assay of the enzymes involved in the esterification of these acids showed that there was negligible formation of the CoA derivatives of succinate and methylmalonate while propionate was readily esterified (Table 8). These results are in contrast to those

TABLE 8

ESTERIFICATION OF PROPIONATE, METHYLMALONATE, AND SUCCINATE BY COA AND ATP AS MEASURED BY HYDROXAMATE FORMATION

Acid	Hydroxamate Formed μMoles
Succinate	~0.1, 0.2
Methylmalonate	~0
Propionate	7.1, 9.5

The system contained (in μmoles): Tris buffer, pH 7.4, 250; CoA, 0.5; ATP, 10; $MgCl_2$, 8; glutathione, 20; hydroxylamine, 1,000; propionate, succinate, or methylmalonate, 100; and enzyme with 4 mg of protein. Final volume, 2.0 ml; incubated 30 min at 30°. Hydroxamic acid was determined by the method of Lipmann and Tuttle.[33]

previously reported by Delwiche *et al.*[34] in which activation of succinate was observed in cell-free extracts of *P. pentosaceum;* however, the preparation of their extracts differed somewhat from that used in the present experiments.

The transfer of CoA between the dicarboxylic acids and propionate was studied by measuring the incorporation of propionyl-C^{14} CoA into dicarboxylic acids via reversible reactions 4, 1, and 2a (Table 9). It is seen that there was considerable

TABLE 9

CoA TRANSFER AS MEASURED BY THE CARBOXYLATION OF PROPIONYL-C^{14}CoA

Substrate	C^{14}-Dicarboxylic Acids Formed* μMoles
Propionyl-C^{14}CoA + methylmalonate	0.001
Propionyl-C^{14}CoA + methylmalonate + ATP + CoA	0.003
Propionyl-C^{14}CoA + methylmalonyl CoA	0.084
Propionyl-C^{14}CoA + succinate	0.060
Propionate-C^{14} + succinyl CoA	0.072

* Calculated as described in footnote of Table 1.
The system contained (in μmoles): potassium phosphate, pH 7, 50; propionyl-C^{14} CoA or propionate-C^{14}, 0.3; methylmalonate, succinate, succinyl CoA, 1; methylmalonyl CoA (active isomer),[34] 0.5; CoA, 1; ATP, 3; enzyme containing 4 mg of protein. Final volume, 0.5 ml; incubated 30 min at 30°.

incorporation of C^{14} from propionyl-C^{14} CoA into the dicarboxylic acids with either methylmalonyl CoA or succinyl CoA. With propionyl-C^{14} CoA and methylmalonate the incorporation of C^{14} was negligible but with succinate it was comparable to that with succinyl CoA. The succinate apparently may be activated by a transfer of CoA from propionyl CoA (reaction 4), as previously reported,[34] but there appears to be little transfer of CoA between propionyl CoA and methylmalonate. Methylmalonate likewise was not activated by ATP plus CoA. The transfer of CoA between propionyl CoA and succinate apparently is reversible, since there was incorporation of C^{14} into the dicarboxylic acids from succinyl CoA and free propionate-C^{14}. These results also demonstrate that methylmalonate must be esterified with CoA before it can be converted to succinate.

There is no *a priori* reason for the transcarboxylation reaction to be confined to the compounds discussed so far. A few preliminary experiments were performed with the extracts of propionibacteria using other acceptors and donors. Since acetyl CoA[35, 36] and butyryl CoA[37] have now been shown to be carboxylated with CO_2 in animal tissues, the ability of these two compounds to act as acceptors in the transcarboxylation reaction was tested. Oxalacetate and acetyl CoA gave rise to malonyl CoA, while oxalacetate and butyryl CoA gave rise to ethylmalonyl CoA. In addition malonyl CoA and ethylmalonyl CoA will transcarboxylate with propionyl CoA, presumably yielding methylmalonyl CoA. Whether or not these transcarboxylations are all catalyzed by the same enzyme is not known at present.

Discussion.—Although the pathway of propionate formation in propionibacteria involves the same compounds as propionate catabolism in animal tissues, there appear to be some differences in the enzyme mechanisms. Tietz and Ochoa[38] reported that the carboxylation of propionyl CoA by pig heart is a one-step reaction involving CO_2 and ATP and that it is catalyzed by an enzyme apparently containing no biotin. However, more recent work has shown that the reaction can be inhibited by avidin.[39] In propionibacteria the carboxylation of propionyl CoA involves a transcarboxylation reaction which also can be inhibited by avidin but which requires no ATP. The fixation of CO_2 into methylmalonyl CoA by propionibacteria, however, does involve ATP. Whether the carboxylation of propionyl CoA with CO_2 is accomplished by formation of E-C_1 from CO_2 or indirectly by carboxylation of catalytic amounts of phosphoenol pyruvate and subsequent

transcarboxylation from oxalacetate is uncertain. Nevertheless, the transfer of a carboxyl group occurs without involvement of ATP and provides a mechanism for the transfer of a carboxyl group from one compound to another without the expenditure of energy. It is possible that a similar transfer may occur during fatty acid synthesis as illustrated in reactions 6 and 7. Since this sequence avoids the formation of free CO_2 and does not require its activation, it may provide more favorable kinetics for the fatty acid synthesis.

$$CH_3COSCoA + \underset{|}{\overset{COOH}{CH_2}}COSCoA \rightarrow \underset{|}{\overset{COOH}{CH_3C}}OCHCOSCoA + HSCoA \quad (6)$$

$$\underset{|}{\overset{COOH}{CH_3C}}OCHCOSCoA + CH_3COSCoA \rightarrow CH_3COCH_2COSCoA + \underset{|}{\overset{COOH}{CH_2}}COSCoA \quad (7)$$

Lynen *et al.*[29] have suggested that in *Mycobacterium* the initial step in CO_2 fixation is a kinase-like reaction whereby ATP reacts with a biotin-enzyme complex to form an ADP $\sim$ biotin-enzyme complex and orthophosphate. The ADP is then displaced "irreversibly" by CO_2. If this mechanism operates in propionic acid bacteria, the loss of activity for CO_2 fixation in our extracts may reflect the loss of the kinase, apparently due to aging of the lyophilized cells. It is possible that the CO_2 fixation occurs by a complex enzyme and that part of the single enzyme becomes inactive. It appears from our results that a C_1-biotin-enzyme may be formed without participation of ATP when the C_1 is transferred from the proper donor. The structural requirements for a donor compound, apparently, are that it have a carbonyl group β to the carboxyl group being donated and adjacent either to another carboxyl group, as in oxalacetate, or to coenzyme A, as in methylmalonyl CoA.

Biotin appears to be firmly bound to the enzyme in some intricate fashion as shown by our failure to inactivate the extracts after several different kinds of treatment. When *P. shermanii* was grown on a medium low in biotin[27] we observed that the extract had low transcarboxylation activity which was not stimulated by the addition of biotin *in vitro*,[40] suggesting that the formation of the biotin complex may not be a simple process.

It was of interest to determine whether transcarboxylation is a widespread reaction, and a cursory examination was made of several preparations of animal tissues. Ammonium sulfate fractions of ox liver, dog heart muscle, dog skeletal muscle, and a preparation of rat liver mitochondria disrupted by sonic treatment were tested for activity. Although all were able to fix CO_2 with propionyl CoA and ATP, only the dog skeletal muscle preparation catalyzed the formation of dicarboxylic acid from propionyl CoA and oxalacetate.[41] The magnitude of the reaction was about one-thirtieth that of the bacterial extract per mg of protein; nevertheless, the results suggest that transcarboxylation is not confined to propionibacteria.

The mechanism of the conversion of methylmalonyl CoA to succinyl CoA is somewhat uncertain. Beck and Ochoa[26] presented evidence from isotope experiments that in animal tissues methylmalonyl CoA transfers a carboxyl group to the β-carbon of propionyl CoA forming succinyl CoA and regenerating a molecule of propionyl CoA. However the mechanism is perplexing for the following reason. Presumably methylmalonyl CoA is the initial carboxylation product because only the α-carbon of propionyl CoA is sufficiently reactive to combine with the C_1 unit. Yet in the isomerase reaction as formulated by Beck and Ochoa the β-carbon of propionyl CoA is now active as an acceptor of a carboxyl group from methylmalonyl CoA. Our studies show that the transfer of a carboxyl group of methylmalonyl CoA to the α-position of propionyl CoA to form a new molecule of methylmalonyl CoA (i.e., exchange) is susceptible to inhibition by avidin (Table 6) while "transfer" to the β-position of propionyl CoA to form succinyl CoA (i.e., rearrangement) is unaffected by avidin (Table 7). Therefore it seems possible that the isomerization may occur by a mechanism differing from the transcarboxylation.

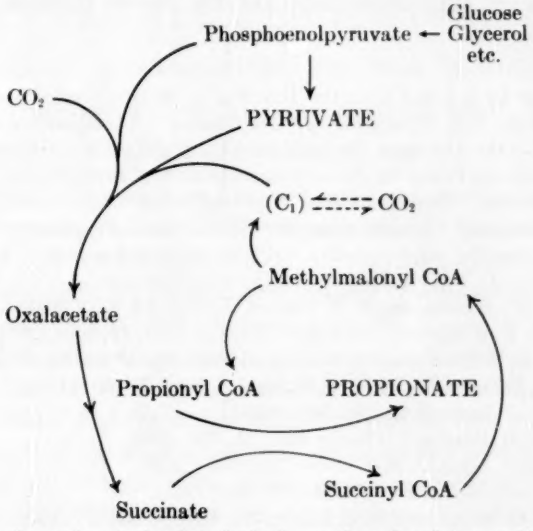

FIG. 1.—Formation of propionate by interlocked reactions involving a C_1 transcarboxylation cycle and a CoA transfer cycle.

The earlier failure to obtain the expected turnover of CO_2 during propionate synthesis *in vivo* in *P. shermanii*[3] now has a demonstrable explanation. The unesterified carboxyl group of methylmalonyl CoA need not be released as free CO_2 but may be transferred to pyruvate with the formation of oxalacetate which can be reduced to succinate, etc. Thus, only catalytic amounts of oxalacetate need be produced by fixation of CO_2. Likewise, coenzyme A may be recycled and only catalytic amounts of the acyl derivatives need be formed by *de novo* synthesis requiring the expenditure of ATP. The transfer of CoA between propionate and succinate has been demonstrated directly by Delwiche *et al.*[34] and indirectly by the present experiments. Indeed, in *P. shermanii* the principal pathway of succinate activation may be by transfer from propionyl CoA.

The scheme shown in Figure 1 is consistent with the results of this study and is

proposed as the pathway of propionate formation from pyruvate in propionibacteria.

Summary.—The pathway of propionic acid synthesis in *P. shermanii* has been studied in cell-free extracts and appears to involve the following reactions. Pyruvate is converted to oxalacetate by the transcarboxylation of a one-carbon unit which is derived from methylmalonyl CoA and which is not in equilibrium with free CO_2. Transcarboxylation is inhibited by avidin and occurs in the absence of fixation of CO_2. Oxalacetate is reduced to succinate which is esterified by the transfer of coenzyme A from propionyl CoA. Succinyl CoA is converted to methylmalonyl CoA by an isomerization which is not inhibited by avidin, malonate, or by treatment with charcoal. The transcarboxylation of methylmalonyl CoA yields propionyl CoA. These interlocked reactions therefore involve a "one-carbon cycle" and a CoA cycle. Thus pyruvate is reduced to propionate with a minimum expenditure of energy and without fixation of CO_2.

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[1] Abbreviations used are CoA (coenzyme A), ATP (adenosine triphosphate), ADP (adenosine diphosphate), ITP (inosine triphosphate), DPNH (diphosphopyridine nucleotide, reduced), TCA (trichloroacetic acid), Tris (tris(hydroxymethyl)methylamine).

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*HOW STABLE IS BALANCED POLYMORPHISM?**

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Natural populations of many species of organisms are polymorphic, i.e., are composed of two or more distinct kinds of individuals. Several genetic mechanisms may participate in the maintenance of polymorphism in populations.[1–5] One of the most widespread and interesting ones is balanced polymorphism. It has long been known[6–8] that if the heterozygotes for a pair of alleles, A_1A_2, are superior in adaptive value to the corresponding homozygotes, A_1A_1 and A_2A_2, natural selection will, in sexually reproducing and cross-fertilizing populations, establish an equilibrium state at which the two alleles, A_1 and A_2, will continue to occur in the gene pool of the population with certain predictable frequencies. This will happen even if one or both of the homozygotes are deficient in fitness, or are inviable, provided only that the heterozygote enjoys the advantages of hybrid vigor.

The mean adaptive value of the population is maximized when stable equilibrium frequencies of the alleles A_1 and A_2 are achieved. Nevertheless, the poorly adapted homozygotes continue to be born generation after generation in populations under balanced polymorphism. In other words, the population fails to reach the level of adaptedness which it would have if it were monomorphic for a genotype with an adaptive value equal to that of the A_1A_2 heterozygotes. Suppose, then, that a mutation produces an allele A_3, such that the fitness of the homozygote A_3A_3 is equal to that of the heterozygote A_1A_2. Natural selection is expected to lead to gradual elimination of A_1 and A_2, and to establishment of a population monomorphic and homozygous for A_3A_3.

These considerations have led to the view[9, 10] that balanced polymorphisms "... are to be regarded as temporary makeshifts that arose in the stress of comparatively rapid evolutionary flux and that are due to be rectified ultimately, when a long-term natural selection repairs its short-term imperfections and miscarriages."

The flaw in the above argument lies in the implied assumption that whatever level of adaptedness may be reached by a heterozygote A_1A_2, it can always be equalled, if not exceeded, by mutations yielding a superior allele A_3. Experimental verification or invalidation of this assumption is evidently difficult, especially if it is not specified just how "temporary makeshift" the balanced polymorphisms are expected to be. Natural populations of many species of Drosophila are balanced polymorphic systems of several gene arrangements, differing in inversions of sections of chromosomes. Although some early speculations which ascribed to inversion polymorphisms an antiquity ascending to Eocene and to Cretaceous times[11] appear to have been not well founded, there is good evidence that balanced polymorphs may not be easily replaceable. Thus in *Drosophila willistoni*, which is a species not closely associated with man, some inversion heterozygotes occur over vast territories, including much of the intertropical zone of the Western Hemisphere. A genetic variant must have existed assuredly for a long time to have spread to, and become incorporated in the gene pools of populations so numerous and so remote.

Laboratory experiments on balanced polymorphisms cover time intervals obviously small compared to the persistence of such polymorphisms in natural populations. There are two considerations, however, which make experimental approaches to the problem hopeful. In the first place, experiments are carried in laboratory environments which are of necessity unlike the natural ones. Experimental populations are thus faced with environmental challenges, to which they may respond by genetic changes adapting them to the experimental environments. A serviceable monomorphism may then be substituted for a balanced polymorphism. Secondly, what is sometimes described picturesquely as a "genetic revolution," may be produced by making experimental populations derived from founders obtained by hybridization between geographically distinct (allopatric) natural populations or races. Segregation and recombination of the genes in which the races differ supply an abundance of genetic variance. Natural selection in the experimental environments is then called upon to construct a new adaptive system, which may or may not include balanced polymorphism.

Many experimental populations of Drosophila flies, polymorphic for naturally occurring inversions, have been studied by various investigators since 1946. In most of these populations apparently balanced equilibria were attained, provided that the foundation stocks of these populations were of geographically uniform origin. It is, of course, arguable that experiments were usually discontinued too soon, before substitution of monomorphism for polymorphism could take place. The few recorded exceptions are therefore interesting. In experiments with *Drosophila pseudoobscura*, a population containing third chromosomes with ST and TL gene arrangements became, in about a year after its foundation, almost monomorphic for ST. It may be noted that TL chromosomes are relatively rare in the natural population from which the foundation stock came.[12]

In an experiment with the same species, in which chromosomes of Texas origin with AR and PP gene arrangements were involved, two experimental populations were started with equal frequencies of the two kinds of chromosomes. Some 605 days later the populations contained about 95 per cent AR and 5 per cent PP chromosomes.[13] It may be noted that in the natural populations of Texas, PP

chromosomes are considerably more frequent than AR chromosomes. This shows how different may be the relative fitness of the same karyotype in natural and in experimental environments. Lewontin's experiments with similar populations brought confirmatory results.[14, 15] But in addition, Lewontin observed that the progress of the selection in the experimental populations was such as to indicate either that the environment, or that the relative fitness of the karyotypes were changing during the experiments. If the latter possibility is correct, it would follow that an evolutionary process was here enacted which led to the abrogation of the balanced polymorphism in favor of monomorphism.

That this is not a general rule for experimental populations of geographically uniform origins, is shown by the data in Table 1. These populations, of *D. pseudo-*

TABLE 1

FREQUENCIES (IN PER CENTS) OF AR CHROMOSOMES IN POPULATIONS OF *Drosophila pseudoobscura* CONTAINING AR AND CH CHROMOSOMES KEPT AT 25°C

		Populations			
Days	Generations	173	176	181	Chi Square
0	0	20.0	20.0	20.0	..
35	1.4	39.3	40.0	40.7	0.12
70	2.8	45.0	48.3	41.3	2.92
105	4.2	50.0	50.3	*	0.15
118	4.7	..	..	55.3	..
140	5.6	60.7	56.7	*	1.21
175	7.0	57.3	..	..	..
264	10.6	..	..	67.7	..
275	11.0	*	66.3	*	1.75
302	12.1	64.3	..	..	..
365	14.6	64.3	71.3	67.7	3.37
488	19.5	..	..	69.0	..
583	23.3	*	78.3	*	8.96
610	24.4	72.7	..	..	..
649	26.0	..	..	66.3	..
790	31.6	74.7	79.7	..	1.83
980	39.2	72.0	..	60.7	8.27

obscura, contained AR and CH chromosomes from southern California (Piñon Flats). In experimental, as well as in natural populations, AR chromosomes have an advantage over CH: an equilibrium is therefore established with AR outnumbering CH. The progress of selection in three populations, Nos. 173, 176, and 181, though they were started at different times, was very similar for at least a year. This is shown by the small chi-square values in the rightmost column in Table 1; (these chi-squares have mostly 2 degrees of freedom; where samples were not taken at similar ages, interpolation was made between the successive samples; such interpolations are marked in Table 1 by asterisks). The experimental populations were kept at 25°C, but the population No. 181 spent several months at 15° when it was about $1^1/_2$ years old, and was later returned to 25°. The sojourn at 15° has apparently altered its genetic properties, and it reached an equilibrium at a lower frequency of AR chromosomes than did populations Nos. 173 and 176. The chi-squares computed for the 583 day and 980 day stages indicate significant differences between No. 181 and the other two populations. All samples contained 300 chromosomes.

The progress of natural selection in populations Nos. 173 and 176, and in No. 181 during the first year of its career, can be accounted for on the assumption that the adaptive values of the three karyotypes were as follows:

AR/AR	AR/CH	CH/CH
0.78	1	0.42

The course of selection predicted on the basis of these values gives a satisfactory fit, thus indicating that the adaptive values of the karyotypes have remained constant during the course of the experiment. The aberrant behavior of No. 181 after it was exposed to changes in the temperature regimen shows, however, that even in populations of uniform geographic origin, genetic variance may be available which may be used to alter the equilibrium position of the balanced polymorphism. And the alteration was, in the present case, towards making the two gene arrangement more nearly equal in frequencies than they were in other populations, not at all towards monomorphism.

Some geographic populations of the sibling species, *Drosophila willistoni*, *D. paulistorum*, and *D. tropicalis* are remarkable in having more than 50 per cent of the individuals heterozygous for certain inversions in their chromosomes.[16] In these populations, one half of the zygotes formed in every generation are handicapped genetically enough to suffer a differential mortality, even under the near-optimal conditions in laboratory cultures. It would seem that natural selection in populations of this sort should be especially likely to lead to a monomorphic condition that would free them from the necessity of paying the "costs" of balanced polymorphism. In August, 1956 we started two experimental populations of D. *willistoni*, using in one (No. 168) progenies of the flies collected some months earlier near Recife, Brazil, and in the other (No. 169) flies from Guaramiranga, Ceará, Brazil.[17] The results obtained are reported in Table 2.

About 60 per cent of the individuals in the Recife population were originally heterozygous for an inversion J, in the third chromosome (IIIJ), which was present in 55 per cent in the Ceará population (Table 2). An inversion E in the right limb

TABLE 2

FREQUENCIES (IN PER CENTS) OF HETEROZYGOTES FOR INVERSIONS IIRE, IIIJ, AND IIIP IN EXPERIMENTAL POPULATIONS OF *Drosophila willistoni* DERIVED FROM PARENTS COLLECTED IN TWO LOCALITIES IN BRAZIL

	Population No. 168 (Recife)			Population No. 169 (Ceara)	
Days	IIRE	IIIJ	IIIP	IIRE	IIIJ
0	14	61	32	65	55
140	30.0	61.5	31.0	53.5	42.5
420	?	59.5	?	59.5	53.5
835	49.0	32.0	11.0	47.0	43.0
1110	51.0	44.0	9.0	50.0	45.0

of the second chromosome (IIRE) was, however, heterozygous in about 65 per cent of the individuals in the Ceará population, but in only 14 per cent at Recife. Moreover, about 32 per cent of the Recife population was heterozygous for the inversion P in the third chromosome (IIIP), which did not occur in Ceará at all. Samples of 200 larvae were examined when the populations were 140, 835, and 1,110 days old from the start. The 420-day samples were obtained by testing the zygotic constitution of 200 adult flies per population[17]; these samples are not entirely comparable with the rest. Table 2 shows that, when the populations were slightly more than three years old, the balanced polymorphism was retained. The frequencies of the heterozygotes have, however, undergone changes; the frequencies

of IIRE and IIIJ heterozygotes decreased somewhat in the Ceará population, as did the IIIJ and especially the IIIP heterozygotes in the Recife population. The frequency of IIRE heterozygotes in the Recife population has, however, increased quite strikingly—it has more than trebled.

An unpremeditated experiment of a much longer duration, up to 211 generations, was performed by maintaining laboratory strains of *Drosophila psuedoobscura*, the wild progenitors of which were heterozygous for certain chromosomal inversions.[18] Although the maintenance of laboratory stocks involves some inbreeding, only 3 of the 27 strains which were originally polymorphic became monomorphic in the course of time. The adaptive advantage of the chromosomal heterozygotes in these populations was evidently strong enough to counteract the effects of inbreeding and genetic drift. At the same time, no well-adapted homozygotes have developed which would have permitted replacement of the polymorphism by monomorphism. It may, of course, be objected that inbred strains do not possess sufficient genetic variance to enable such evolutionary changes to take place. This stricture does not apply to experiments described below.

Chromosomally polymorphic experimental populations of Drosphila have been made using for the foundation stocks hybrids between strains of flies coming from different geographic regions. Such populations of geographically mixed origin often behave quite differently from those of geographically uniform origin.[13, 19, 20] The process of natural selection in populations of mixed geographic origin is characteristically erratic, and often quite different in replicate experiments. The balanced chromosomal polymorphism is sometimes retained and sometimes lost. The selective processes which are enacted in such populations lead to compounding of new adaptive systems from genetic fragments yielded by the hybridization of the geographic races. The replacement of the balanced polymorphisms present in the parental races by chromosomal monomorphisms certainly proves that acquisition in evolution of a balanced polymorphic condition need not be irreversible; the population is not, so to speak, committed to remain polymorphic forever. On the other hand, retention of the polymorphism shows that it may be advantageous even when the adaptive system in a population undergoes reconstruction. It has been cogently argued[14, 15] that balanced polymorphism is most likely to be retained in populations living in variable environments, and replaced by monomorphism in uniform environments. Although the environmental conditions in the experimental populations are not quite uniform, yet they are almost certainly more nearly so than in the natural habitats of Drosophila.

Levine and Beardmore[21] have maintained an experimental population of *Drosophila pseudoobscura* of mixed geographic origin (California + Mexico) for about 53 months (66 generations). In about 20 generations from the start, the population established an equilibrium of about 30–35 per cent CH and 65–70 per cent AR chromosomes, and kept it till the end of the experiment, with occasional fluctuations up to 40 per cent and down to 25 per cent of CH.

We have considered most suitable for testing the stability of balanced polymorphism the populations of *Drosophila paulistorum* and *D. willistoni* which contain, in nature, more than 50 per cent of heterozygotes for certain inversions (see above and the references 16 and 17). Two experimental populations of *D. paulistorum* were set up, with the foundation stocks of mixed geographic origin. In about 10

months, corresponding to 12–15 generations, the balanced polymorphism broke down and the populations became virtually monomorphic.[17] A different outcome was, however, observed in the populations Nos. 178 and 179 (Table 3), the founda-

TABLE 3

FREQUENCIES (IN PER CENTS) OF HETEROZYGOTES FOR INVERSIONS IIRE AND IIIJ IN EXPERIMENTAL POPULATIONS OF *Drosophila willistoni* OF HYBRID ORIGIN

Days	Population No. 178		Population No. 179	
	IIRE	IIIJ	IIRE	IIIJ
0	41.75	52.0	41.75	52.0
300	27.5	45.0	34.0	38.0
400	33.75	35.0	38.0	34.0
640	40.5	42.0	45.5	35.0
820	45.5	45.5	52.0	34.5
980	44.0	46.0	53.5	37.0

tion stocks of which were hybrids between the experimental populations of *D. willistoni* Nos. 168 and 169 (see Table 2).

As shown in Table 3, the frequencies of the heterozygotes for the inversion IIRE decreased at first in both populations; instead of progressing towards monomorphism, the heterozygotes became, however, more frequent again, and, in fact, reached frequencies higher than they had in the foundation stocks. The incidence of the heterozygotes for the inversion IIIJ also declined in both populations. In No. 179 a new equilibrium was established at a level lower than in the foundation stock; in No. 178 the frequency of heterozygotes diminished at first, but later recovered a part of this initial loss, and became stabilized. These populations apparently did not possess genetic materials from which monomorphic genetic systems conferring high adaptedness could be built.

Conclusions and Summary.—As a method of adaptation, balanced polymorphism is a costly one, since it entails production of some relatively unfit homozygotes. Balanced polymorphism is nevertheless widespread in natural populations of Drosophila and probably in other sexually reproducing organisms as well. There is every reason to think that the chromosomal balanced polymorphisms in Drosophila are stable. This is shown by the wide geographic distribution and the apparent antiquity of many natural polymorphisms, and also by their retention in experimental populations in which opportunities are offered for adaptive reconstruction of the genetic system. On the other hand, establishment of balanced polymorphism in a population is not an evolutionary blind alley, as shown by the emergenec of secondary monomorphisms in some experimental populations.

From a long-range view, the evolutionary advantages of genetic systems based on balanced polymorphisms may lie, as suggested by Lerner[22] and others, in their great adaptational plasticity and in the possession of the property of genetic homeostasis. It may also be that the fitness conferred upon their carriers by heterozygosis for balanced supergenes is not easily equalled in homozygotes. How important are balanced polymorphisms in the genetic systems of man and of other higher organisms with limited fecundity is an open question.[23] Chromosomal inversions of the kind known in Drosophila are unlikely to be common in mammals, but other genetic mechanisms which form and maintain balanced supergenes may exist. The losses of the homozygotes of lower fitness are more difficult to put up with, but they can be reduced by development of numerous heterotic supergenes acting like multiple alleles of single loci.

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*THE OCCURRENCE OF PARTIALLY FERTILE TRIPLOID METAFEMALES IN DROSOPHILA MELANOGASTER**

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Bridges[1] was the first to discover the existence of triploid females (3X3A) in *Drosophila melanogaster* and the occurrence of intersexes (2X3A), superfemales (3X2A), and supermales (1X3A) among their offspring. It was also in these triploid lines that Bridges[2] discovered the occurrence of tetraploid females (4X4A). From his studies of these sex types Bridges concluded that sex determination in *Drosophila* was an example of genic balance, the X chromosomes carrying a preponderance of female determining factors, the autosomes carrying a preponderance of male determining factors. The sexuality of an individual could be expressed by the ratio between the number of X chromosomes and the number of sets of autosomes it possessed, an X:A ratio of 1.0 being the norm for femaleness, an X:A ratio of 0.5 being the norm for maleness. Values between these two figures produce intersexes, values above or below these two figures produce the supersexes.

The terms "superfemale" and "supermale" are actually inappropriate since the excess of genetic determinants for femaleness or maleness results developmentally in weak individuals with sterile, underdeveloped gonads. The prefix "meta" (be-

yond), suggested by Dr. Curt Stern,[3] will be used hereafter in place of "super" to signify phenotypes beyond the range of normal maleness or normal femaleness.

Individuals with X chromosome-autosome combinations other than those described by Bridges are possible and their characteristics could be predicted on his genic balance theory. One such type would have 4 X chromosomes and 3 sets of autosomes. By analogy with the term "triploid intersexes" used for 2X3A individuals, and by contrast to the 3X2A "diploid" metafemales, 4X3A individuals will be termed triploid metafemales. The triploid metafemale X:A ratio of 1.3 lies between the normal female ratio of 1.0 and the diploid metafemale ratio of 1.5, and the triploid metafemale characteristics described below are in fact intermediate.

L. V. Morgan[4] probably observed triploid metafemales among the offspring of a tetraploid female. She described two sterile flies which she believed were 4X3A on the basis of their Bar phenotype but referred to them as intersexes, possibly because their triploidy and wing disturbances make them look similar to extreme female type intersexes.

This paper presents genetic and cytological proof of the occurrence of triploid metafemales and describes their general characteristics.

Origin and Phenotype.—A total of 49 triploid metafemales were recovered from a cross in which the parent triploid females carried attached-X chromosomes, a free X chromosome, and a Y chromosome. The attached-X chromosomes were homozygous for the genes *yellow*[2], *scute*, *apricot*, and *echinus*, and the free X chromosome carried the markers *yellow*[31d], *scute*[8], *diminutive*, and *Bar*. Multiple inversions in the free X chromosome (written invX in Table 2) almost entirely prevented crossing over with the attached-X chromosomes. The inversions covered the region from the tip of the X chromosome to a point just left of *Bar*. The male parent carried an X chromosome marked with *yellow* and a Y chromosome carrying a short translocation of the tip of the X chromosome with the wild type allele of yellow (the $sc^8 \cdot$ Y chromosome). The cross (Exp. I) can be written out as follows:

$$\underline{y^2\ sc\ w^a\ ec}/y^{31d}\ sc^8\ dm\ B/\mathrm{Y};\ \text{3A females by } y/sc^8\cdot\mathrm{Y};\ \text{2A males.}$$

(In a slightly different experiment (Exp. II) the triploid females carried the $sc^8 \cdot$ Y chromosome, the male carried a normal Y chromosome, and the male X chromosome was marked with *yellow* and *white*.)

Nine per cent of the offspring of this cross were observed to come from eggs in which secondary nondisjunction of the X chromosomes had occurred. One of the products of nondisjunction is an egg containing both the attached-X and free X chromosomes, and these eggs frequently receive two sets of autosomes. Such eggs have an equal chance of being fertilized by an X bearing sperm or Y bearing sperm. Thus triploid metafemale zygotes would be expected to occur as frequently as the exceptional class of triploid female zygotes and would contain all the X chromosomes involved in the cross.

The constitution of the two types just described plus the constitution of the regular class of triploid females is as follows:

Triploid metafemale $\underline{y^2\ sc\ w^a\ ec}/y^{31d}\ sc^8\ dm\ B/\mathrm{Y};$ 3A

Phenotype: yellow[2], Bar

Exceptional triploid female $\underline{y^2\ sc\ w^a\ ec}/y^{31d}\ sc^8\ dm\ B/\mathrm{Y};$ 3A

Phenotype: yellow[2], scute, Bar

Regular triploid female $y^2\ sc\ w^a\ ec/y$; 3A

Phenotype: yellow[2]

The scute classification is not reliable but the Bar phenotype is markedly different in triploid females and triploid metafemales. *Bar* is semidominant, the presence of wild type alleles causing the eye to be larger. In the series *B* male, $B/+$ female, and $B/+/+$ triploid female the eye size increases in easily distinguished steps until in triploid metafemales ($B/+/+/+$) *Bar* is detectable only as a small indentation in the anterior portion of the eye.

Aside from the genetic markers used, triploid metafemales have a distinctive phenotype. They have the stout bristles, large eye facets, and coarse wings characteristic of triploid females, but their eyes are more bulging and the general body build is smaller. Characteristics they share with diploid metafemales are the rough eyes, the narrow wings with cut and excised inner margins, and the frequent absence of various bristles. In coloration and lack of sex combs they resemble normal females. The smaller body build and the degree of wing abnormality were quite slight in a few cases.

Genetic and Cytological Proof.—Five of the triploid metafemales were fertile. Since each of the X chromosomes in the parental cross was distinctive, one should be able to recover the attached X's, the inverted X, and the normal X among the offspring of any given triploid metafemale. This expectation was successfully tested for three of them, the two others producing too few offspring for an adequate sample. None of the triploid metafemales collected in Exp. II received the $sc^8 \cdot$ Y-chromosome from the triploid female parent and presumably the same rule is true for Exp.I where the Y chromosome in the triploid female parents was not genetically marked. That the autosomes were triploid, and not tetraploid, is indicated by the type of offspring recovered from the fertile triploid metafemales, i.e., 1 triploid metafemale, 17 triploid females, 1 intersex, and 29 diploids (23 females, 6 males). Tetraploid females would be expected to give mostly triploid females or intersexes.

FIG. 1.—Metaphase oogonial plate from a triploid metafemale (4X3A). The drawing is slightly schematic. The X chromosomes are in the lower half of the figure with the two groups of autosomes in the upper left and upper right respectively.

An ovary smear was made from one of the sterile triploid metafemales. The ovaries were small but had a few almost mature eggs. Several mitotic metaphases were found clearly showing the presence of two dot shaped chromosomes, two groups of 3 V-shaped chromosomes, two rods, and a V-shaped chromosome whose arms had the same morphology as the rods (Fig. 1).

Viability and Fertility.—It was pointed out earlier that triploid metafemales and exceptional triploid females should be produced in equal frequencies. Since triploid female viability is high we may estimate the viability of triploid metafemales by comparing the numbers recovered in the two groups. This estimate, together with other information on fertility is given in Table 1. This table indicates that environmental differences between Experiments I and II had a strong influence on viability. There is also variation in viability between genotypes. Some of the parents in the two experiments carried the dominant autosomal genes, *Curly* and

Lobe, and triploid metafemale viability among the *Curly-Lobe* containing offspring (using Curly-Lobe triploid females as standards) was only 4 per cent. The over-all 25 per cent viability of triploid metafemales in these experiments may be contrasted with the 1 per cent viability of diploid metafemales collected in the same experiments.

Five triploid metafemales produced 1, 6, 11, 14, and 16 offspring respectively, and many more laid eggs. This is in contrast with the complete sterility of diploid metafemales. It is interesting to note that the 11 per cent of eggs developing to adults recorded in Table 1 is much higher than that found by Beadle and Ephrussi[5] for eggs produced by diploid metafemale ovaries transplanted into diploid females (30 offspring from several thousand eggs).

Chromosome Segregation and Crossing Over.—It was possible to determine the chromosomal constitution of all the offspring from triploid metafemales and hence, of the eggs from which they were derived. The array of egg types recovered is presented in Table 2. This table shows that the two free X chromosomes had both passed to the pole opposite the attached-X chromosomes in two thirds of the 48 eggs. Another striking feature of the table is that most of the attached-X chromosomes recovered came from 2A eggs, while most of the free X chromosomes recovered came from 1A eggs.

TABLE 1

VIABILITY AND FERTILITY OF TRIPLOID METAFEMALES

	Number of Exceptional Triploid Females	Triploid Metafemales: Viability 2nd Column / 1st Column % — Number	Triploid Metafemales: Viability 2nd Column / 1st Column %	Triploid Metafemales: Per Cent That Laid Eggs*	Triploid Metafemales: Range in Number of Eggs Produced	Triploid Metafemales: Per Cent Eggs That Developed to Adults†
Exp. I	89	36	40	24 (±7)		
Exp. II	105	13	12	54	1–150	11
Total	194	49	25			

* A complete record was not kept in Exp. I and the ±7 indicates the maximum and minimum number of triploid metafemales that could have laid eggs.

† Based on 42 adults (including 19 diploid females, and 5 males) emerging from an approximate egg count of 386.

TABLE 2

DISTRIBUTION OF EGG TYPES RECOVERED FROM TRIPLOID METAFEMALES

	X Chromosomes						
Autosomes	$\overset{\wedge}{XX}$	$\overset{\wedge}{XX}$, invX	$\overset{\wedge}{XX}$, X	X	invX	X, invX	0
1A	3	..	..	7	5	13	1
2A	14	2	1	..	..	2	..

It was also possible to test most of the chromosomes for recombination between the normal free X and the attached-X's. Eleven recombinants between *yellow* and the centromere were discovered out of 31 such chromosomes tested, a rate much higher than that found by Beadle and Ephrussi (1 recombinant out of 26 chromosomes) for the same type of crossover in offspring recovered from eggs produced by the transplanted diploid metafemale ovaries.

Discussion of these interesting data will be deferred to a more comprehensive study of chromosome segregation in different types of triploids.

Summary.—Genetic and cytological proof is given for the occurrence of triploid metafemales containing 4 chromosomes I, 3 chromosomes II, 3 chromosomes III, and 2 chromosomes IV. Phenotypically the triploid metafemales resemble triploid

females in their large eye facets, stout bristles and coarse wings, and resemble diploid metafemales in their rough eyes and narrow wings with cut and excised inner margins.

Viability of triploid metafemales is much higher than that of diploid metafemales and they are also partially fertile. Five of the 49 triploid metafemales collected were fertile, these 5 producing a total of 48 offspring. The partial fertility of triploid metafemales yielded data on chromosome segregation and crossing over frequency.

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THE BEHAVIOR OF NONHOMOLOGOUS CHROMOSOMAL ELEMENTS INVOLVED IN NONRANDOM ASSORTMENT IN DROSOPHILA MELANOGASTER

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Highly nonrandom assortments of nonhomologous chromosomes in *Drosophila melanogaster* females have been reported.[1, 2] That the unexpected distribution of chromosomes is the consequence of directed segregations during meiosis can be established by the equality of reciprocal classes. Other phenomena, such as preferential inclusion of certain chromosomes into the egg nucleus or differential mortality of zygotes, would not be expected to produce equal reciprocal classes. Since the behavior of the chromosomes during meiosis in the female of *Drosophila* is not amenable to cytological analysis, it may only be inferred that the segregation of nonhomologues to opposite poles in excess of 50 per cent is preceded by associations between them. The interpretation of the data presented here will be based on this inference.

Studies of the conduct of chromosomal elements involved in nonrandom assortment were undertaken with the purpose of establishing a set of rules that govern nonrandom assortments. Specifically, this report is directed toward providing answers to the following questions: (1) Is the Y chromosome a necessary component of a system that results in high frequencies of nonrandom assortment? (2) Does competition occur between different elements for association with a nonhomologue? (3) Are there different degrees of attraction between different non-

homologues? (4) Are proximal heterochromatic regions the only areas of the chromosomes determining nonhomologous associations?

General Methods.—Nonrandom assortments of nonhomologous chromosomes occur when two or more nonhomologues are present during meiosis without adequate pairing partners. Homologues may be rendered inadequate for normal pairing by structural rearrangements, or extra chromosomes may be added to the normal complement of the female to provide chromosomes unpaired with their homologues. Thus the females in which the phenomenon was originally observed carried an extra Y chromosome and a heterozygous 3;4 translocation, T(3;4)86D.[2] The presence of the translocation reduces pairing between the free fourth chromosome and its homologue involved in the translocation. In this situation, the Y chromosome and the fourth chromosome pass to opposite poles with a frequency of 83 per cent; the Y chromosome and the translocation pass to opposite poles with a frequency of 62 per cent; the fourth chromosome and the translocation assort randomly. Presumably, it is the T_4 fragment (that portion of the translocation that carries the centromere and proximal heterochromatin of the fourth chromosome up to 101F and the euchromatin of 3R distal to 86D) rather than the T_3 fragment (that portion of the translocation that carries all of the third chromosome up to the breakpoint at 86D and the fourth chromosome distal to 101F) that is involved in the nonrandom assortment. This conclusion is based partially on Brown's analysis[3] of the behavior of a translocation, T(3;4)A9 that has a break at 87E and partially on data that will be presented.

In the analysis of the pairing behavior of the chromosome, the fourth chromosome and the T_4 chromosomal element, the frequencies of association between all pairs of these chromosomal elements in the presence and in the absence of the third element were calculated. In making these calculations, we have assumed that association of two elements is followed by their regular separation during the first meiotic division, whereas their failure to associate is followed by random assortment. Since half the products of random assortment are nondisjunctional for the elements, association (a) may be calculated as one minus twice the frequency of nondisjunction (n), i.e., $a = 1 - 2n$.

The third element is assumed to be unavailable for nonhomologous association and "absent" from the system when it has been supplied a structurally identical element that saturates its pairing ability. Thus the T_4 was eliminated from the system through the use of a homozygous translocation and the free fourth chromosome by the addition of another free fourth chromosome. The use of an XX female eliminated the Y chromosome.

A factor in measuring the association between the T_4 and any other element is the limited availability of the T_4, owing to its high frequency of pairing with its homologous region in the right arm of the normal third chromosome. If the normal third chromosome is replaced by the third chromosome carrying a short inversion in the region that is homologous to T_4, it can be shown that the availability of the T_4 is greatly increased and marked shifts in the association relations among the Y, the fourth chromosome, and T_4 occur.

To learn if the Y chromosome is a necessary component of a system showing a highly nonrandom assortment of nonhomologous chromosomes, we performed a set of experiments in which the Y chromosome was replaced by a free X duplication.

Materials.—The translocation used in all the experiments, T(3;4)86D, is viable in the homozygous condition. It carries a closely linked marker, bx^{34e}, and a loosely linked marker e^4. Genetic analysis has shown that bx^{34e} separates from the translocation by crossing over with a frequency of about 1 per cent.

Three different Y chromosomes, Y^{C-S}, sc^S·Y, and YB^S have been used in the experiments.[2, 4, 5] The method for following the unmarked Y^{C-S} by its suppressing action on the position effect of In(2R)bw^{VDE1} is given in the earlier paper on nonrandom assortment.[2]

The free X-chromosome duplication that was used for the experiments is Dp(1;f)3.[6] This is a grossly deleted chromosome that carries the centromere and proximal heterochromatin of the X chromosome, including the loci of bb^+ and $su\text{-}f^+$, and the distal euchromatic tip, including the loci of y^+ and sc^+.

Inversion (3R)Vno was used to disrupt pairing between T_4 and its homologous region in the nontranslocated third chromosome. The inversion extends from 89E to 97A on the salivary gland chromosome map according to Nicoletti's analysis.[7] The inversion is inseparably associated with a reliable, easily classified dominant mutant, *Vno* (*Vein off*). The mutant phenotype is a gap in the second longitudinal wing vein.

Results and Interpretation.—The raw data and the calculated segregation frequencies are shown in Tables 1 and 2. The association frequencies that are derived from the segregation frequencies by the use of the formula, $a = 1 - 2n$ are presented in Table 3. The interpretation will most often be in terms of the association frequencies.

It should be pointed out that, if the normal third chromosome can co-orient T_3 and T_4 in such a manner that they pass to the same pole more often than to opposite poles, a bias will exist favoring recovery of products of T_4-3 association as opposed to T_4-Y and T_4-4 associations. Preferential recovery of the products of T_4-3 associations should lead to the concommitant preferential recovery of

TABLE 1

SEGREGATION FREQUENCIES OF PAIRS OF ELEMENTS IN "ABSENCE" OF EXTRA ELEMENT

A. Gametes from y/y/sc^S·Y; T(3;4)86D, bx^{34e} e^4/+; ci^D/spa^{Cat} ♀ ♀ mated to y; bx^{34e} ♂♂

Free fourth chromosome	3;4	Y;T(3;4)/4	T(3;4)/4	Y; 3; 4
ci^D	260	380	453	676
spa^{Cat}	321	366	499	637
Totals	581	746	952	1313

sc^S·Y − T_4 segregation = 63.1 per cent. One 3; ci^D/spa^{Cat} gamete recovered.

B. Gametes from normal 3. T(3;4)86D, bx^{34e} e^4/+; ci^D ♀ ♀ and T(3;4)86D, bx^{34e} e^4/In(3R)-Vno, Vno; ci^D ♀ ♀ mated to bx^{34e} ♂♂

Third chromosome	T(3;4)	3;4	T(3;4)/4	3
Normal 3	917	969	464	217
In(3R)Vno	256	247	3	0

4-T_4 segregation in presence of normal 3 = 67.0 per cent;* 4-T_4 segregation if In(3R)Vno is present = 98.8 per cent.*

C. Gametes from y^2/y^2/Dp(1;f)3, y^+; T(3;4)86D, bx^{34e} e^4/T(3;4)86D, bx^{34e} e^4/ci^D ♀ ♀ mated to y ♂♂

Dp(1;f)3	Dp(1;f)3;ci^D	no extra elements	ci^D
167	3	4	173

Dp-4 segregation = 98.0 per cent.

* These frequencies were calculated by doubling the number of triplo-4 flies and omitting the haplo-4 flies because the latter are inviable.

TABLE 2

SEGREGATION FREQUENCIES OF PAIRS OF ELEMENTS IN PRESENCE OF EXTRA ELEMENT

A. In(3R)Vno present

1. Gametes from X/X/YB^s; T(8;4)36D, bx^{34e} e^4/In(3R)Vno, Vno; ci^D ♀ ♀ X wild type ♂ ♂
2. Gametes from y^2 v/y^2 v/Y^{c-s}; bw; T(3;4)86D, bx^{34e} e^4/In(3R)Vno, Vno; ci^D ♀ ♀ X In(2R)bw^{VDe1}/SM1 ♂ ♂
3. Gametes from y^2 v/y^2 v/Dp(1;f)3, y^+; T(3;4)86D, bx^{34e} e^4/In(3R)Vno, Vno; ci^D ♀ ♀ X y; bx^{34e} ♂ ♂

		T(3;4)	Vno; 4	T(3;4)/4	Vno
1.	YB^s	2	193	0	4
	no YB^s	190	7	207	0
2.	Y^{c-s}	4	86	0	39
	no Y^{c-s}	121	7	184	0
3.	Dp(1;f)3	19	135	0	36
	no Dp(1;f)3	136	23	331	0

1. YB^s-T_4 segregation = 98.3 per cent; YB^s-4 segregation = 52.9 per cent; 4-T_4 segregation = 48.7 per cent.*
2. Y^{c-s}-T_4 segregation = 97.8 per cent; Y^{c-s}-4 segregation = 61.2 per cent; 4-T_4 segregation = 41.0 per cent.*
3. Dp-T_4 segregation = 95.3 per cent; Dp-4 segregation = 72.2 per cent; 4-T_4 segregation = 32.5 per cent.*

B. Inversion(3R)Vno absent

Gametes from X/X/YB^s; T(3;4)86D, bx^{34e} e^4/+; ci^D ♀ ♀ X bx^{34e} ♂ ♂

Y chromosome constitution	T(3;4)	3;4	T(3;4)/4	3
YB^s	205	121	9	157
no YB^s	153	233	347	8

YB^s-T_4 segregation = 66.1 per cent; YB^s-4 segregation = 76.6 per cent; 4-T_4 segregation = 50.1 per cent.

* These frequencies were calculated from the no Y classes in order to eliminate the highly inviable haplo-4 class

TABLE 3

ASSOCIATION FREQUENCIES BETWEEN Y OR DP, THE FOURTH CHROMOSOME, AND THE T_4 FRAGMENT

Asrociation → / Extra Element →		Y or Dp and 4 T_4	Per cent	Y or Dp and T_4 4	Per cent	4 and T_4 Y or Dp	Per cent	T_4 and 3 Y or Dp Per cent
Extra Element Absent	1. In(3R)Vno Absent	Y^{c-s}	84.2	sc^s.Y	26.2		32.8	(64.8)†
		Dp	96.0					
	2. In(3R)Vno Present						97.6	
Extra Element Present	3. In(3R)Vno Absent	YB^s	54.4	YB^s	33.2	YB^s	2.8	(64.8)†
		Y^{c-s}	66.4	Y^{c-s}	23.0	Y^{c-s}	1.2	(64.8)†
	4. In(3R)Vno Present	YB^s	5.8	YB^s	96.6	YB^s	− 2.6*	
		Y^{c-s}	22.4	Y^{c-s}	95.6	Y^{c-s}	−18.0*	
		Dp	44.4	Dp	90.6	Dp	−35.1*	

* A negative value indicates both elements pass to the same pole in excess of 50 per cent.
† Inferred from the data.

products of Y-4 associations. The extent of the co-orientation of the T_3 and T_4 by the normal third chromosome cannot be evaluated from the available data, but after a consideration of Brown's results,[3] we do not believe that it is great enough to alter any of the interpretations.

The dispensability of the Y chromosome: In previous experiments, a Y chromosome was always used as one of the elements involved in highly nonrandom assortment of nonhomologues.[1, 2, 3] To test the possibility that the Y chromosome was a necessary component in such a system, we substituted the deleted X chromosome, Dp(1;f)3 for the Y chromosome in females that were homozygous for T(3;4)86D and carried a free fourth chromosome marked with ci^D. The progeny of such females reveal that the duplication and the free fourth chromosome associate with a frequency of 96 per cent. The Y chromosome is obviously not unique in its ability

to associate with the fourth chromosome, and in fact the duplication appears to associate with a fourth chromosome even more frequently than does a Y.

Competition of elements: Competition between three elements may be demonstrated when the removal of one of them results in an increased association between the other two. A comparison of the frequencies of association of the Y^{C-S} and the fourth chromosome in the presence and absence of T_4 (Table 3, lines 1 and 2) shows that association between the Y and fourth chromosomes significantly increased (66.4 versus 84.2 per cent) in the latter case; also the frequency of association of the fourth chromosome and T_4 is higher in the absence than in the presence of a Y chromosome (32.8 versus 1.2 per cent). Both these results indicate that the elements are in competition.

Competition among three elements may also be demonstrated when an increase in the availability of one leads to an increase in the association between that element and a second element and simultaneously leads to a decrease in the association between the second element and a third element. When a comparison of the frequency of association between the Y and T_4 in the presence and absence of the fourth chromosome is made no significant change is observed (26.2 versus 23.0 or 33.2 per cent). At this point it is necessary to consider that the T_4 fragment possesses a sizable homologous pairing region in the distal euchromatin of 3R. If both 3R and the Y are competing for T_4, then the availability of T_4 for associations with the Y may be limited by the competitive efficiency of 3R. Reduction of this efficiency might be expected to result in radical changes in the associations of the three elements. The use of In(3R)Vno to disrupt pairing between the T_4 fragment and 3R does in fact increase the availability of T_4 as shown by the increase in association between T_4 and the fourth chromosome from 32.8 per cent in the absence of the inversion to 97.6 per cent in its presence (Table 3, lines 1 and 2). A comparison of the associations between the three elements in the absence and presence of In(3R)Vno (Table 3, lines 3 and 4) shows that the increased availability of the T_4 fragment when In(3R)Vno is present leads to increases in Y-T_4 association from 33.2 to 96.6 per cent (YB^S) and from 23.0 to 95.6 per cent (Y^{C-S}). Furthermore, the association betwen the Y chromosome and the fourth chromosome is concommitantly decreased from 54.4 to 5.8 per cent (Y^{C-S}) and from 66.4 to 22.4 per cent (YB^S). These shifts in association frequencies again demonstrate competition.

Degrees of attraction between nonhomologues: The attraction of two different elements for a nonhomologue may be judged only when both these elements are equally available for associations. This condition is realized when the Y, the T_4, and the fourth chromosome as well as the inversion are present (Table 3, line 4). In this situation, the YB^S associates with one nonhomologue, the T_4, seventeen times as frequently as it associates with the other nonhomologue, the fourth chromosome; in the case of the Y^{C-S} and the Dp(1;f)3, a fourfold and a twofold preference for the T_4 over the fourth chromosome, respectively, is observed. Clearly then, in every case, different elements exhibit different degrees of attraction for a nonhomologue.

These data provide information on whether the segregations can be best interpreted on a bivalent univalent model or on a trivalent model. According to the bivalent-univalent model, the sum of the pairwise associations of any element with the other elements cannot exceed 100 per cent. An association of 100 per cent

indicates that the element is associated with one of its possible associates in every oocyte. A sum of associations in excess of 100 per cent means that an element is associating and separating from two of its possible partners in some oocytes, and consequently a trivalent model must be postulated.

Although the total YB^S associations (102 per cent) do not significantly exceed 100 per cent and hence indicate a bivalent-univalent type of association with the fourth chromosome and the T_4, the total Y^{C-S} associations (118 per cent) and, more markedly, the total Dp(1;f)3 associations (135 per cent) do significantly exceed 100 per cent and indicate separation from a trivalent association. Contrary to expectation a one-armed element, Dp(1;f)3, appears capable of directing two other elements to the same pole.

The role of proximal heterochromatin in nonhomologous associations. Regions of shared homology in the proximal heterochromatin have been postulated as responsible for associations between nonhomologues.[9] A favorable opportunity for testing whether such associations are exclusively determined by proximal heterochromatin is afforded in the present experiments. The two elements that compete for associations with the Y chromosome or the duplication, i.e., the fourth chromosome and the T_4, carry the same proximal heterochromatin of the fourth chromosome up to salivary chromosome map region 101F. Although the dividing line between heterochromatin and euchromatin in chromosome four has not been precisely defined, the available evidence places it close to 101F.[10, 11] In any case, if heterochromatin is present beyond this point, it is the fourth chromosome that carries it and the T_4 fragment that lacks it. The euchromatic compositions of the T_4 and the fourth chromosome, however, are entirely different since T_4 carries only euchromatin of 3R. Since the Y chromosome and the duplication associate much more frequently with the T_4 than with the fourth chromosome, the preference for association with the T_4 must be attributed to the difference of its euchromatic composition from that of the fourth chromosome.

General Conclusions.—Answers to the questions posed and certain other conclusions concerning the behavior of nonhomologous elements involved in nonrandom assortments in the female of Drosophila can be given: (1) Nonrandom assortments of nonhomologues may be induced in high frequencies by the presence in the genome of two or more nonhomologous elements that lack homologous pairing partners. (2) The equality of reciprocal classes that result from these assortments establishes that they are the result of meiotic segregations. (3) The Y chromosome is not a necessary component of this system. (4) When more than two such elements are present, competition for associations occur among them. (5) Different elements have different degrees of attraction for a nonhomologue. (6) The proximal heterochromatic regions of chromosomes are not entirely responsible for nonhomologous associations.

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*THE EFFECT OF ACRIDINE DYES ON MATING TYPE FACTORS IN ESCHERICHIA COLI**†

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In *Escherichia coli*, female strains are designated as F^-; male strains are of two kinds, designated F^+ and Hfr, respectively. The male determinant in Hfr strains behaves as a chromosomal factor allelic to F^-.[1–3] In F^+ strains, however, maleness is determined by a factor "F," with remarkable properties, notably its easy, contagious transmission to F^- cells.[4–6] Cells carrying F can be disinfected by treatments with cobalt ion and with acridine dyes.[7, 8] These properties support the conclusion that F is a plasmid, an extrachromosomal particle, which is readily transferred during mating contacts. It has been suggested that Hfr strains represent the incorporation of F as an element of the chromosome.[1, 2, 4, 5, 9] Jacob and Wollman introduced the term "episome" for a plasmid that has a facultative association with the chromosome.[9] The present paper reports further evidence for this conception, namely on the mechanism by which F is eliminated by the acridine dyes.

Materials and Methods.—Two acridine dyes, proflavine (PF, 2:8-diaminoacridine), and acridine orange (AO, 2:8-*bis*Dimethylaminoacridine) were used. Stock solutions containing 100 μg per ml of PF or 500 μg per ml of AO in water were autoclaved and stored in the dark for periods up to a week.

EM-sugar agar[11] was used as a selective medium. To grow on this medium, a recombinant must be prototrophic and also be able to ferment the sugar, e.g., lactose. Nutrient medium used for acridine treatment consisted of Difco peptone, 10 and Difco meat extract, 10 gm per liter. The pH of the medium was adjusted with sodium hydroxide solution using the Beckman pH meter. Difco penassay broth (Antibiotic assay medium number 3) was used routinely for bacteriological work.

Strains of *E. coli* used in these experiments are mutants derived from strain K-12. The production, origin, and characteristics of these mutants are summarized in Table I. The strain used for acridine treatment was mainly W6.

Recombination technique: Overnight cultures of tester strains are streaked on EM-sugar medium and they are cross-brushed against one loopful of the culture being tested. Recombinants arise at the junction of the two cultures only in compatible combinations.[4]

Acridine method: An overnight F^+ culture is diluted to 10^4 cells per ml in a

TABLE 1

A SUMMARY OF THE STRAINS USED

Strain Number	Genotype	Original Strain	Reference
W6	F^+M^-	K-12	[4]
W1895	Hfr_1M^-	W6	Type Hfr[5]
W2979	$F^-Mal_1^-Xyl_2^-Gal_2^-Ara_2^-$		J-11 (supplied by Dr. Cavalli)[1]
W3133	F^-Lac^-		Ann Cook[10]
W4164	F^+Lac^-	W3133	Obtained by F infection
W4171	F^+M^-	W1895	Spontaneous reversion (given by Dr. A. Novick)
W4399	F^rM^-	W1895	F-refractory[15] (selected in soft agar);[16] Low fertility X F^- and does not infect F^- to produce F^+

nutrient broth (pH 7.6) containing 20 micrograms per ml of acridine orange (abbreviated AO-20) and incubated overnight at 37°C. The use of acriflavine for the removal of F has been briefly reported in an earlier publication.[8] AO has proved to be more useful than acriflavine, proflavine, or acridine yellow by virtue of its low toxicity.

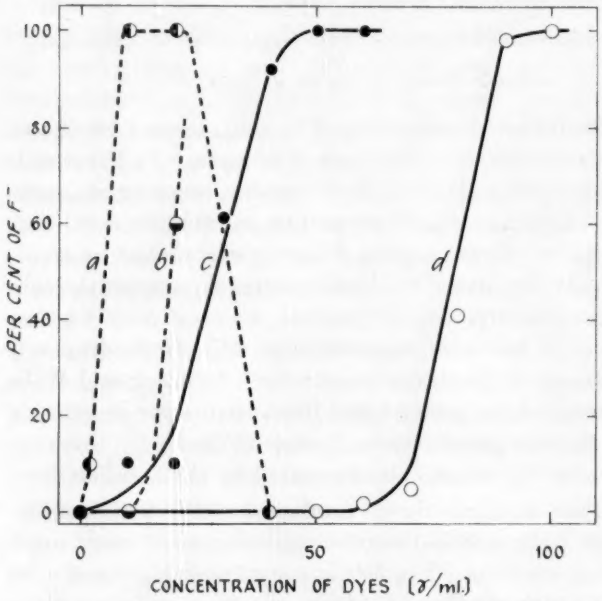

FIG. 1.—Effect of pH on the effective concentration of acridines. (*a*) Proflavine, pH 7.65. (*b*) Proflavine, pH 7.20. (*c*) Acridine orange, pH 7.60. Acridine orange, pH 7.20. An overnight culture of W6 was inoculated into broth (10^7 cells/ml, pH 7.20 or pH 7.6) containing the indicated dye and incubated at 37° C for 20 hours before plating.

The F^- clones obtained in these experiments have been repeatedly studied. The F^- strains so obtained are genetically stable for this mating type, like the other F^- reported. If any F^+ cells had reappeared in the F^- culture by spontaneous mutation, they would be amplified by cross-infection during repeated passages.

Experimental Results.—1. Environmental effects on the action of acridines: The conversion of sex-compatibility after growth in acridine broth was observed by a standard test of sex-compatibility on isolated clones. The results are generally expressed in terms of the conversion fraction, i.e., the proportion of clones, after treatment which have become stably F^-. The pH influences the effect of the dyes; for example, AO-50 gives 100 per cent F^- at pH 7.6 but none at pH 7.2. Generally speaking, the minimum effective concentration of acridine is low at a high pH and vice versa (Fig. 1). All the colonies formed in the untreated control remained F^+.

Proflavine exerts its action at lower concentrations than that of AO at the same pH. However, the rate of conversion is decreased at higher (bacteriostatic) concentrations of PF. These pH effects indicate that either the drug cation or an

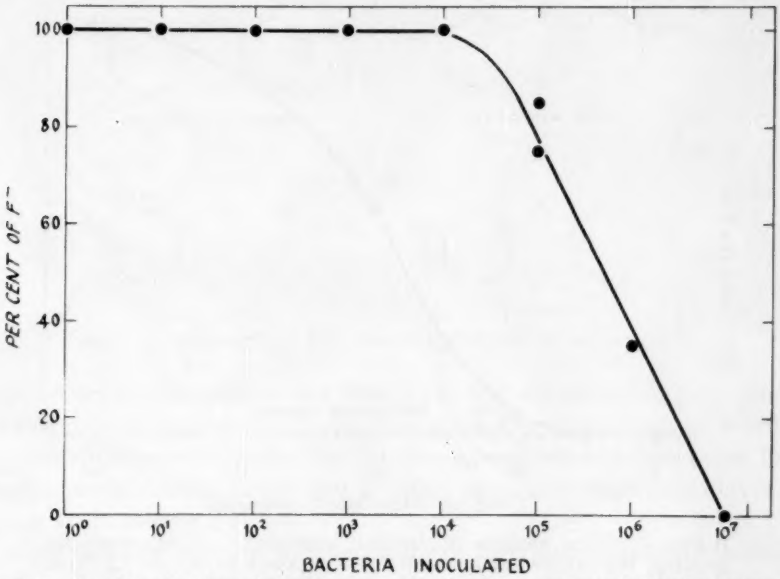

FIG. 2.—Effect of inoculum size on conversion of sex-compatibility. Various dilutions of an overnight culture of W6 were inoculated into broth containing AO (pH 7.6, 20 μg./ml) and incubated overnight at 37°C.

anionic bacterial receptor is effective in the elimination of F. The same relationship between bacteriocidal action of acridine ion and pH effect has been extensively studied by Albert.[12] The inoculum size of F^+ cells also influences the rate of conversion, which decreases with larger inocula (Fig. 2). With an inoculum of about 1 cell per ml, 100 per cent F^- can be obtained in nutrient broth at pH 7.6 with AO 10 to AO-20. The lowest concentration at which any effect was noted was AO-1 to AO-5.

The rate of elimination is reduced at lower temperatures and was essentially zero at 5°C (Table 2). The viability of the cells obtained from the treated culture and the untreated culture at low temperature is the same.

TABLE 2

EFFECT OF TEMPERATURE ON CONVERSION OF SEX-COMPATIBILITY FROM F^+ TO F^-

	Treated with AO		Untreated Control	
Temperature (°C):	37	5	37	5
Total colonies tested:	110	110	110	110
F^- obtained:	91	4	0	3
Per cent of F^-:	82.7	3.6	0.0	2.7

10^7 cells per ml of an overnight culture of W6 were inoculated into nutrient broth pH 7.6 and treated for about 20 hours with 50 micrograms of acridine orange, or without it as control.

Figure 3 shows the conversion fraction in treated cultures plated on EMB lactose agar at various times. F^- clones first appear after a lag of 1 to 2 hours, and their incidence then increases rapidly. The concentration of AO, pH of the medium, and cultural age of the treated cells influence the rate of conversion and the time for removal of F.

Acridines are ineffective on cells growing in a synthetic medium.[13] However, peptone 0.02 per cent, restored the effect (Table 3). A systematic analysis of

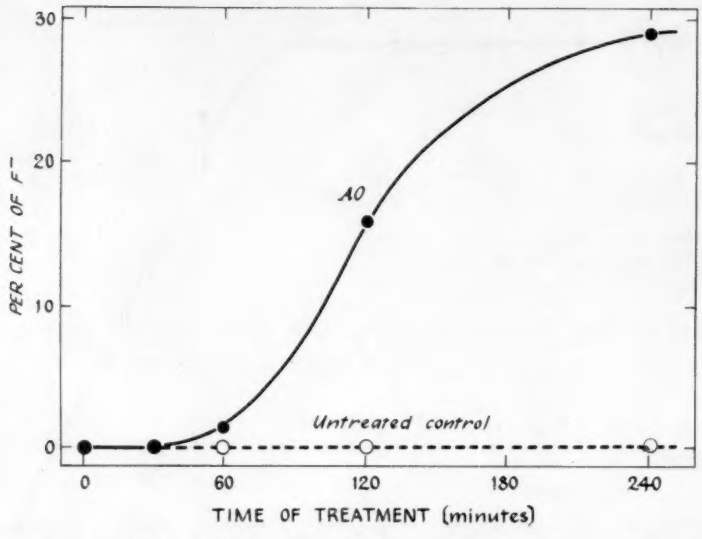

FIG. 3.—Time analysis of acridine treatment. An exponentially growing, W6 culture was inoculated into penassay broth (pH 7.2) with or without AO (36 μg./ml). The F^+ cultures (10^7 cells/ml), were shaken gently at 37°C. Samples were diluted and plated on EMB agar. The following morning single colonies were tested for their compatability. The figure summarizes five experiments. About 2,000 colonies from untreated controls were all F^+.

likely constituents of complex media showed the necessity for the simultaneous presence of several co-factors: amino acids plus a nuclein base. AO eliminated F from F^+ bacteria in a defined medium containing the following supplements: serine, aspartic acid, isoleucine, valine, and cytosine. The significance of these co-factors for the cure of maleness remains for future studies. The specific supplements required for growth of auxotrophic mutants were also essential for F elimination.

TABLE 3

CO-FACTOR FOR REMOVAL OF F BY AO

Minimal plus Indicated Supplement	Per Cent of F^- Obtained	
	W6 (M^-)	W4164 (Prototroph)
No supplement	0	0
Peptone* plus AO	100	...
Mixture†	71	100
" minus AO	0	0
" minus serine	4.1	10.1
" " cytosine	3.7	0
" " methionine	0	33.4

* 200 micrograms per ml.

† Concentration of each supplement: cytosine 100, serine 20, aspartic acid 20, isoleucine 20, valine 20, methionine 20, AO 7.5 (micrograms/ml).

Overnight cultures of W6 and W4164 were washed with sterilized water, treated in several media, [illegible] on EMB agar, and their compatibility tested by the cross-brushing method. The experimental cond[illegible] were as follows: inoculum size about 10^4 cells per ml, time of treatment 20 to 24 hours at 37°C.

Methylene blue and thionine which are structurally analogous (Fig. 4) to AO antagonize the elimination of F but not the inhibition of growth by AO. These dyes alone have no dramatic activity on F^- disinfection; F^- clones have appeared infrequently in treatments with methylene blue. Albert[12] has summarized analogous examples of "therapeutic interference" in the typanocidal action of these dyes.

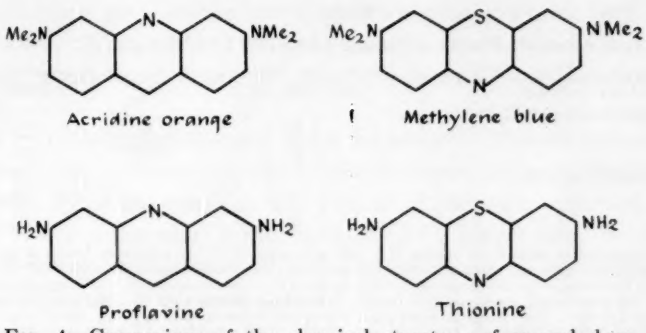

FIG. 4—Comparison of the chemical structure of several dyes.

Propamidine isethionate (Baker and May Ltd., 4:4′-diamidino-diphenoxypropane di-(β-hydroxyethane sulfonate)) is another chemotherapeutic agent which supposedly reacts with nucleic acids. At 50 micrograms per ml in penassay broth it was found to produce about 15 per cent F^- after overnight treatment, and is therefore a weak agent affecting F.

It is concluded that acridine mainly produces its effect on actively multiplying cells: in the absence of cell multiplication, i.e., with large inocula, low temperature, nutritional limitation, or bacteriostasis by excess dye, there is little or no loss of F.

2. Mode of action of acridine: The increase in the proportion of F^- after acridine treatment could be attributed in principle either to mutagenic effects of acridine dyes or selective enrichment of F^- cells already present among the F^+ population. No differential in growth of F^+ vs. F^- cells could be found even remotely approaching the dramatic differences needed to account for complete conversions (see Table 4). However, the most decisive experiments concerned the fate of small numbers of cells inoculated in AO medium.

TABLE 4

RECONSTRUCTION EXPERIMENT

	Input Mixtures				Mixed Cultures after Incubation			
	$Lac^+ F^-$	$Lac^+ F^+$	$Lac^- F^-$	$Lac^- F^+$	$Lac^+ F^-$	$Lac^+ F^+$	$Lac^- F^-$	$Lac^- F^+$
A Treatment	0.329 (W2979)	0	0	0.671 (W4164)	0.471 (W2979)	0	0.529 F-elimination	0
Untreated Control	0.363 (W2979)	0	0	0.637 (W4164)	0.246 (W2979)	0.152 F-infection	0	0.602 (W4164)

Overnight cultures of W4164 (F^+) and W2979 (F^-) were mixed and about 10^4 cells/m l inoculated into broth, pH 7.6, with or without AO, and incubated overnight at 37°C. The proportion of Lac^+ and Lac^- bacteria in the mixed cultures was diagnosed on EMB lactose agar before and after the treatments; the F status of samples of isolated colonies was tested by crosses against a standard F^- strain (see Materials and Methods). The figures in this table show the fraction of colonies in each class.

The mutagenic effect of AO was tested more directly by cultivating one or two cells in a microdroplet of penassay broth, with or without acridine dye, in an oil-chamber as described by Lederberg.[14] The microcultures were then allowed to grow for several generations. Each microclone was transferred to EMB lactose agar by a capillary pipette and plated out to give single colonies arising from individual cells. The colonies were then tested for sex-compatibility. The entire clone was plated; thus, differential growth of spontaneous F^- mutants could alter the proportion of F^- progeny within a clone but should not affect the number of

TABLE 5

INDUCTION OF F^- FROM SINGLY ISOLATED F^+ CELL AND F^+ CELLS

Number of Cells in Drop Initially	Number of Drops with Induced F^-	Number of Drops Tested
Acridine-treated cell(s)		
1	3*	25
2	5†	20
Untreated Control		
1	0	25
2	0	20

An exponential culture of strain W6 was grown at 37°C in penassay broth with gentle shaking. This culture contained 10^9 cells per ml. Single cells were then isolated in microdroplets by a simple modification of De Fonbrune's oil chamber method.[14] Droplets were dispensed by free hand manipulation from a suspension containing 10^6 cells per ml. with or without AO-40. The droplets were then examined microscopically, and those which were verified to contain precisely one cell or two cells then recorded. They were incubated at 37°C for 4 hours. At the end of this time, the cells that had grown in each drop were counted, then collected by a capillary pipette and spread on EMB agar. All the colonies formed on the agar were tested for sex-compatibility. If one or more F^- colonies were observed, this was recorded as "containing induced F^-."

By Fisher's exact test, P = 0.0056.

Total numbers of F^- cells in "induced drops" were:

* 1,1,1.

† 20,2,4,1,2.

and the total cell numbers of those induced droplets were:

33,20,10; 72,10,34,11,71, respectively.

clones in which F^- progency are found. As shown in Table 5, AO increases the proportion of F^--containing clones from 0/45 in controls to 8/45 in the treated series. This supports the conclusions that AO induces the loss of F from growing F^+ cells.

3. The action of AO on various male strains: A wide variety of F^+ strains mutants of *E. coli* K-12 have been tested. The markers such as nutritional requirements, drug resistance, lysogenicity, phage resistance, sugar fermentation seem to have no bearing on the effect of AO nor are they altered by the treatment. However, Hfr males differ markedly from F^+. For example, Table 6 shows a result of

TABLE 6

ACCESSIBILITY OF TWO STATES OF F IN VARIOUS STRAINS TO DISINFECTING ACTION OF ACRIDINE TREATMENT

	W6 F^+	W1895 Hfr	W4171 F^+ Reversion from W1895	W4399 F-Refractory
Can infect standard F^-	+	−	+	−
Mode of inheritance of maleness	Nonsegregational	Segregational	Nonsegregational	Segregational
Compatibility with standard F^-	+	+++	+	±
Compatibility status affected by acridine treatment	yes	no	yes	no

acridine treatment on four male types all derived from W6. Mutants which show chromosomal segregation and non-infectivity of the male character are not sensitive to the disinfecting action of acridine dyes. This inaccessibility is observed both in stable Hfr and the F^r mutants (summarized by Lederberg and Lederberg).[15] Hirota and Iijima[8] reported that AO gave F^- types not only from F^+ but also from Hfr_2.[6] However, this culture has proven to be extremely mutable and spontaneously produces many F^+ reversions. The F^- obtained from cultures labelled Hfr_2 can be accounted for by their origin from such F^+ reversions. The F^+ state of the mutable Hfr_3[2] is also accessible to this treatment. Stable Hfr strains, e.g., Hfr_1 have not been influenced by exposure to AO. On the other hand, the other

mutants which maintain a contagious F are readily disinfectable by this treatment. F elimination by AO thus differs from the correlated selection of highly motile F^- bacteria in soft agar, which selects F^- not only from F^+ but also from stable Hfr strains.[16]

Discussion.—In this phenomenon, many hypotheses may be considered. From the above experiment, however, it seems difficult to escape the conclusion that induction of F^- from F^+ is the result of the loss of F particles endowed with genetic continuity. Whether the relative multiplication of F is decreased, or the F particles are agglutinated by combination with the acridinium cation, growing cells exposed to AO eventually produce bacteria without F.

Stability of F^- means a permanent loss of F, and conversely the stable maintenance of the F^+ trait and the remarkable infectivity of F in usual media signify that F multiplies faster than the host cell. The transfer of F unlinked to the host chromosome, and the non-segregational uniform inheritance of F in the cross $F^+ \times F^-$,[4-6] would most logically correspond to cytoplasmic transfer of the male determinant.[17, 18] That is, the factor in an F^+ cell is a plasmid.[8, 17, 18] Other plasmids, such as the respiratory factors in yeast, kinetoplasts in trypanosome are also known to be accessible to acridine treatment.[19-22]

On the other hand, maleness in Hfr cells is inherited chromosomally as a gene linked to certain markers in genetic recombination tests.[1-3] Correlated with this is the fact that Hfr males are resistant while infective F^+ males are accessible to the acridine treatment (Table 6). These facts suggest that in Hfr strains this male determinant is bound to the particular chromosome site, and thus accounts for the non-infectivity and segregational inheritance of maleness, and its inaccessibility to the disinfecting action of acridine dyes.

This situation parallels the lysogenic system of bacteria in which there are two states, vegetative phage, free in the cytoplasm and prophage, bound to the chromosome. The term episome has been used for this type of particle.[9]

Acridine dyes are reported to cure virus infection, produce defective virus, and induce virus mutants respectively.[23-27] However, they do not affect the chromosomally bound prophage of temperate phages.[28] The detailed mechanism of action is, however, not known. These dyes do possess the unique property of straining certain nucleic acid-containing particles, nuclei, and the other particles of living cells, in several organisms.[29] Nucleotides, nucleic acid, and polyadenylic acid form complex salts with acridine dyes.[30, 31] The effect of acridine orange on the function of DNA as a primer for its enzymatic replication has not been studied.

The co-factors required for F-disinfection may act in part by supporting the growth of the host cell in its particulate components in the presence of AO. The F particle itself may have a negative charge, so that acridine exerts its effect only as a cation.

Summary.—Acridine orange converts F^+ (male) clones of *E. coli* into stable F^- (female) forms. The main factors which affect the rate of conversion of F^+ to F^- are the concentration of acridinium ions in the treating medium and the growth of the treated cells in the presence of the dye. Furthermore, the accessibility of F to acridine treatment depends upon the state of F in the host cell.

Acridine orange increases the frequency of conversion from F^+ to F^- directly without appreciable selective growth. The conversion from F^+ to F^- is irreversible,

as expected for the loss of a genetic particle. Hfr males are resistant to the disinfecting action of acridine dyes. These results are well accounted for by the dual nautre of F, chromosomal F, and plasmid F.

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*EVOLUTION AND PTERIDINE METABOLISM IN THE GENUS DROSOPHILA**

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Introduction.—The characterization of the major pteridines contained in *Drosophila melanogaster*,[1, 2, 3] coupled with the use of paper chromatographic techniques for the separation of these compounds, has made possible a relatively reliable visual method for their identification. The pteridine nature of the *Drosophila* red eye pigments has been demonstrated,[1] and they have been found to consist of three orange-fluorescing compounds.[4] Investigations of eye-color mutants of *D. melanogaster*[5] have indicated a metabolic relationship between the red eye pigments and the other pteridines found in *Drosophila*. This information, plus information from a comparative study of fluorescing compounds from other species of *Drosophila*,[6] has prompted a study to reveal some of the major changes in the accumulation of these compounds that have occurred through evolution in the genus *Drosophila*.

The present knowledge of pteridine metabolism in *Drosophila*, chiefly from work on *D. melanogaster*, can be summarized briefly. The compound, 2-amino-4-hydroxypteridine, is converted to isoxanthopterin by the enzyme xanthine dehydrogenase. This same enzyme controls the production of uric acid from hypoxanthine. The mutants *maroon-like* and *rosy*[2] lack this enzyme activity and accumulate hypoxanthine, 2-amino-4-hydroxypteridine, sepiapteridine and biopterin.[7, 8] The mutant *sepia* lacks any red pigments and accumulates the yellow pigments, sepiapteridine and compound A[2, 5] in very large quantities, suggesting a precursor accumulation such as is common in biochemical mutants. It has been shown by *in vivo* labeling experiments that 2-amino-4-hydroxypteridine is converted directly into isoxanthopterin and that the compound is apparently not converted to any other pteridine.[8] The structures, and the known and postulated reaction sequences, of the pteridines found in *D. melanogaster* are given in Figure 1.

The work of Rasmussen[6] has shown that *Drosophila* species fall into two major categories. The more primitive species[9] have red pigments (drosopterines) in the body and the more advanced forms lack them or have them present at very low levels. The work in *Drosophila* taxonomy agrees and indicates that the males of the more primitive forms have colored testes and the more advanced forms have colorless testes. Exploratory determinations have clearly shown that the pigments in the testes are chromatographically and electrophoretically identical with the eye pigments. Close relationship between the red pigments in the eyes and those in the testes is indicated also by the fact that in *D. virilis*, a species having red testes, mutants which lack pigments in the eye also lack them in the testes.

These relationships suggest that one of the major evolutionary changes of pteridine metabolism in the genus *Drosophila* has been restriction of drosopterine accumulation from occurring in both eyes and testes to occurring in the eyes only. Since it is possible that this restriction could have been accomplished in several different ways, it seems probable that part of the chromatographic variation be-

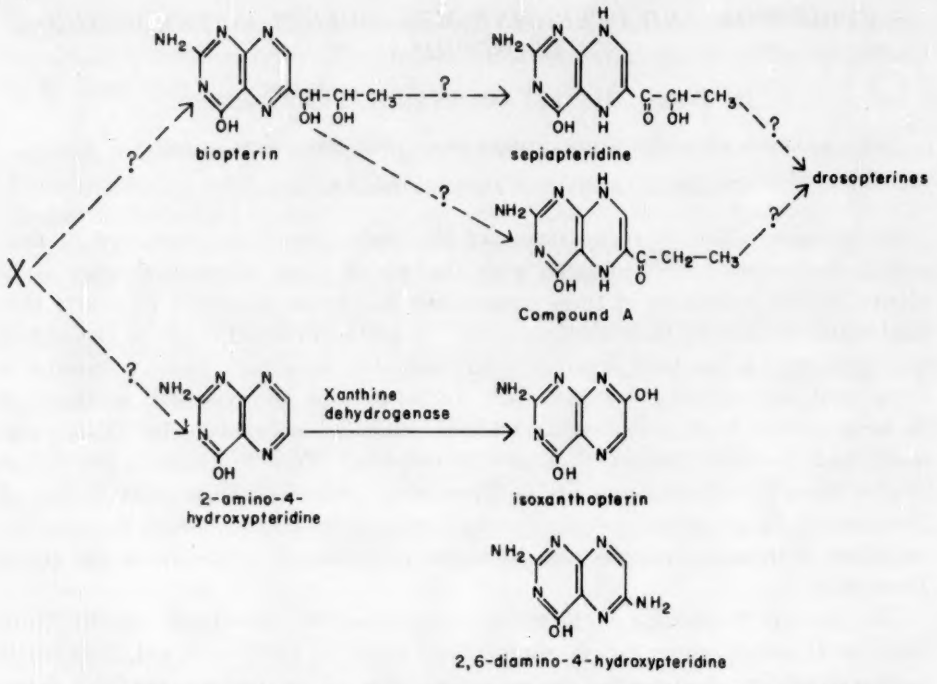

FIG. 1.—Structures and postulated reaction sequences of the pteridines in *Drosophila*.

tween species is a reflection of such change. So long as these metabolic changes were of adaptive value, resulting forms of metabolism would be retained and be observable in some of the present species of *Drosophila*. It may be predicted that a phylogenetic sequence of species should show various stages in the elimination of drosopterine accumulation by the testes. Comparisons between accumulations by various species and by various mutants of *D. melanogaster* allow interpretations regarding the types of metabolic change which have occurred during the evolution of pteridine metabolism in the genus *Drosophila*.

Materials and Methods.—Adult male *Drosophila* aged for ten days were used throughout this study. All data were obtained from laboratory stocks raised on cornmeal-agar or on banana-agar food. The stocks were raised and chromatography was done at 20° ± 1°C. A simple method of descending paper chromatography was used with consistently repeatable results. The aged males were etherized and then weighed on a Cahn Electrobalance. An attempt was made to maintain a constant sample weight of four milligrams. The flies were decapitated and the bodies were placed in boiling, distilled water for two minutes. After thorough blotting, the boiled bodies were smashed compactly on a sheet of Whatman No. 1 chromatographic paper. The heads were smashed directly on the paper without boiling. The sheets were equilibrated in the darkened chamber in the presence of 7 per cent aqueous ammonia. The solvent used was made up of *n*-propanol, 8 parts; ammonium hydroxide (S.G., 0.89), 1 part; distilled water, 3 parts (v/v/v). After 12 hours' irrigation, the papers were removed to dry in a dark room.

The dried chromatograms were viewed with a Mineralight ultraviolet lamp

(principal emission 365 mμ). Initial identification of compounds was made by running known samples on the same chromatogram with samples of *D. emarginata*. After the identity of the various spots was determined, *D. emarginata* was run as the standard for comparison. It contained all of the major compounds seen, and when these did not separate on any particular run the chromatograms were discarded and the work repeated. Samples of isoxanthopterin, 2-amino-4-hydroxypteridine, and biopterin were obtained from Dr. H. S. Forrest for comparison and identification. The mutant *sepia* of *D. melanogaster* was used as the source of sepiapteridine. Uric acid and xanthurenic acid were obtained commercially (California Corporation for Biochemical Research, Los Angeles, California). The major unknown fluorescent material observed on these chromatograms was a greenish-yellow spot having a slightly higher R_f than isoxanthopterin. It may be 2,6-diamino-4-hydroxypteridine of Van Baalen and Forrest[10] or xanthopterin. The area of the chromatogram containing sepiapteridine also contains other yellow-fluorescing compounds. One of these materials is probably riboflavin. It was not always possible to determine the amount of sepiapteridine in flies which contained appreciable amounts of these other yellow-fluorescing materials. To detect uric acid and xanthurenic acid, the chromatograms were sprayed with N,2,6-trichloroquinoneimine.[11] Uric acid was investigated because of its metabolic relationship to isoxanthopterin through the enzyme xanthine dehydrogenase.

Amounts of the various pteridines were estimated visually. Five categories were used for initial recording of data. Weight of each sample was then taken into consideration, and the final data were recorded in three categories, with "+" indicating trace amounts, "++" indicating moderate amounts, and "+++" indicating large amounts. Final data are estimates of the amounts expected from a four-milligram sample of each species.

Table 1 lists the number of species examined in each group. For brevity, only two or three species from each group will be reported here. When species within a group differed in the amounts of compounds accumulated, the extreme representatives are given in the data. If members of a group were homogeneous, two random species are reported. A large number of species from the repleta group are included since they provide an example of the kinds of divergence found. Well over 150 species have been observed, and the complete data will be reported elsewhere.

The phylogeny followed here is, for the most part, taken from Patterson and Stone.[12] Changes suggested by other workers[13, 14] have been followed in some places. The phylogeny of Wasserman[15] has been followed for members of the repleta group. Data from groups of uncertain relationship will not be reported here, since they add little to interpretations regarding changes in pteridine metabolism with evolution. The major phylogenetic relationships within the genus are reasonably well established, but any phylogeny must be considered to be speculative and its tentative nature recognized.

Since the primary goal of this study was to determine the chromatographic patterns of as many forms as possible, only one sample from one strain of each species was used. Buzzati-Traverso[16, 17] has demonstrated the constancy of chromatographic patterns within a genotype, and he has confirmed the observation of Hadorn and Mitchell[5] that the patterns of *D. melanogaster* are not affected by die-

TABLE 1

NUMBER OF SPECIES EXAMINED FROM EACH OF THE GROUPS STUDIED

Systematic Category	Number Studied
Genus *Drosophila*	
Subgenus *Dorsophila*	1
Subgenus *Drosophila*	
annulimana group	1
calloptera group	3
canalinea group	3
carbonaria group	1
cardini group	10
dreyfusi group	3
funebris group	4
guarani group	4
guttifera group	1
immigrans group	3
melanica group	4
mesophragmatica group	2
pallidipennis group	1
quinaria group	11
repleta group	29
robusta group	2
rubrifrons group	2
testacea group	1
tripunctata group	16
virilis group	9
Subgenus *Hirtodrosophila*	3
Subgenus *Pholadoris*	7
Subgenus *Sophophora*	
bromeliae group	2
melanogaster group	11
nannoptera group	1
obscura group	10
saltans group	3
willistoni group	8
Total	156

tary or environmental conditions. In the course of this work, *D. emarginata* was chromatographed approximately sixty times. The chromatographic pattern obtained was constant within the limits of observation, and it is considered that the chromatographic data reported here adequately represent the characteristics of each species.

Results.—The results from heads and bodies of representative species from the genus *Drosophila* are listed in Tables 2 and 3. Only two compounds from the heads showed marked quantitative variation. Otherwise there were only slight random and gradual changes in the amounts of the various compounds (Table 2). An unknown blue-fluorescent spot (BI) was seen primarily from species in the saltans and willistoni groups of the subgenus *Sophophora*. Outside of this subgenus, this compound was seen in a few scattered species from groups belonging to the quinaria branch of the subgenus *Drosophila*. This compound is not a pteridine, but nothing else is known regarding its identity. The other compound, xanthurenic acid (XIC), was present in the heads from most species in the genus. However, the majority of the species belonging to the virilis-repleta branch of the subgenus *Drosophila* lacked this compound. All species contained drosopterines in the heads.

The accumulations of pteridines in the bodies of the flies varied markedly. In order to represent the data from Table 3 more clearly, the results have been shown

TABLE 2

AMOUNTS OF VARIOUS COMPOUNDS ACCUMULATED IN THE HEADS OF REPRESENTATIVE SPECIES FROM THE GENUS *Drosophila*

	DRO	ISO	XAN	SP	AHP	BIO	URC	XIC	BI
Subgenus *Drosophila*									
Virilis group									
americana	+++		++	++	++	+	++		
borealis	+++		++	++	++	+	++		
Repleta group									
aldrichi	+++		++	+	+	+	++		
mulleri	+++	+	++	+	++	++	++		
hydei	+++		+	+		++			
fulvimaculoides	+++		++	++	+	++	++		
Carbonaria group									
carbonaria	+++		++	++	++	++	++		
Melanica group									
melanica	+++	+	++	++	++	++	++		
euronotus	+++	+	++	++	++	+	++		
Robusta group									
lacertosa	+++		+	+	+	+	+		
robusta	+++	+	++	+	++	+	++		
Quinaria group									
occidentalis	+++	+	++	+	+	+	++	++	
quinaria	+++	+	++	+	+	+	++	+	
munda	+++	+	+	+	+	+	++	++	
Guarani group									
griseolineata	+++	+	++	++	++	+	++	+	
subbadia	+++	+	++	+	++	+	++	+	
Cardini group									
cardini	+++		+++	++	++	+	+	+	
dunni	+++	+	++	++	++	++	++	++	
Tripunctata group									
setula	+++		++	++	++	+++	+++	+++	
unipunctata	+++	+	++	++	+	+	+++	+++	
mediostriata	+++	+	++	++	++	+	++	++	
Guttifera group									
guttifera	+++		++	+	++	+	++	++	
Subgenus *Sophophora*									
Obscura group									
affinis	+++		++	++	++	++	++	++	
pseudoobscura	+++		++	++	++	+	++	++	
Nannoptera group									
nannoptera	+++	+	++	++	+++	+++	++	++	
Saltans group									
emarginata	+++	+	+++	+++	++	+++	++	+	++
prosaltans	+++		+++	++	+++	++	++	++	++
Willistoni group									
nebulosa	+++	+	++	+	++	+	+++	+	+
equinoxialis	+++		+++	++	++	++	+++	+	++
Melanogaster group									
seguyi	+++		++	++	++	++	++	++	
ananassae	+++		+++	++	++	++	++	++	

Key to abbreviations and symbols used in Tables 2, 3, 4, and 5.
DRO = drosopterines; ISO = isoxanthopterin; XAN = unknown yellow-green fluorescing compound; SP = sepiapteridine; AHP = 2-amino-4-hydroxypteridine; BIO = biopterin; URC = uric acid; XIC = xanthurenic acid; BI = unknown blue-fluorescing compound. + = trace amounts, ++ = moderate amounts, +++ = large amounts.

diagrammatically and placed in phylogenetic sequence in Figures 2 and 3. Figure 2 shows the results for drosopterine and sepiapteridine (colored pteridines), and Figure 3 shows the results for 2-amino-4-hydroxypteridine, biopterin and isoxanthopterin (noncolored pteridines). The primitive species from each subgenus (members of the obscura and virilis groups) uniformly contain both sepiapteridine and drosopterines in the body. More highly evolved forms from any of the four major phylogenetic lines included here show a great reduction in the amounts of these compounds. *D. lacertosa* and *D. quinaria* have been included as representa-

TABLE 3

AMOUNTS OF VARIOUS COMPOUNDS ACCUMULATED IN THE BODIES OF REPRESENTATIVE SPECIES FROM THE GENUS *Drosophila*

	DRO	ISO	XAN	SP	AHP	BIO	URC
			Subgenus *Drosophila*				
Virilis group							
americana	+++	+++	++	+++	+	+	+++
borealis	++	+++	++	+++	++	+	+++
Repleta group							
aldrichi	++	++	+	++	+	+	+++
mulleri		+++	++	+++	++	++	++
hydei		+++			++	+	
fulvimaculoides		+			+	+	
Carbonaria group							
carbonaria	++	++	+		++		+
Melanica group							
melanica	+	+++	+	++	++	+	+
euronotus		++	++	+++	++	+	+++
Robusta group							
lacertosa	+	+	+	++	+	+	++
robusta		+	+	+			++
Quinaria group							
occidentalis	++	+++	++	+++		+	++
quinaria	+	++	+	+	+	+	++
munda		++	+	++		+	++
Guarani group							
griseolineata	++	+	+	++		+	++
subbadia		++	+			+	++
Cardini group							
cardini		+++	+	+++		+	++
dunni		++				+	+
Tripunctata group							
setula		+	+	+		+	++
unipunctata		++	+	+		+	+
mediostriata	+	++				+	++
Guttifera group							
guttifera		+++			+	++	
			Subgenus *Sophophora*				
Obscura group							
affinis	++	++	+	++		+	++
pseudoobscura	+++	++		++	++	+	++
Nannoptera group							
nannoptera	+++	+++	++	+++	++	++	++
Saltans group							
emarginata	+	++	+	+++	+	+++	++
prosaltans		+++	+	+	+	++	++
Willistoni group							
nebulosa	++	+++	+	+++	++	+	+
equinoxialis		+++			+		+
Melanogaster group							
seguyi	++	+++		++	++	+	+
ananassae		+++		+	+	+	+

tives of the type having these compounds reduced but not absent. *D. hydei*, *D. dunni*, and *D. equinoxialis* are examples of types where both compounds have been reduced to such low levels that their presence could not be demonstrated by our methods.

The accumulation of the three noncolored pteridines is rather uniform throughout the genus (Fig. 3). However, the quantity of these compounds is reduced in some of the more highly evolved forms. Other differences between groups are also evident. Species of the virilis group accumulate 2-amino-4-hydroxypteridine in moderate amounts, and they generally accumulate more of this than of biopterin. Species of the quinaria group accumulate little or no 2-amino-4-hydroxypteridine and have amounts of biopterin equivalent to that seen in members of the

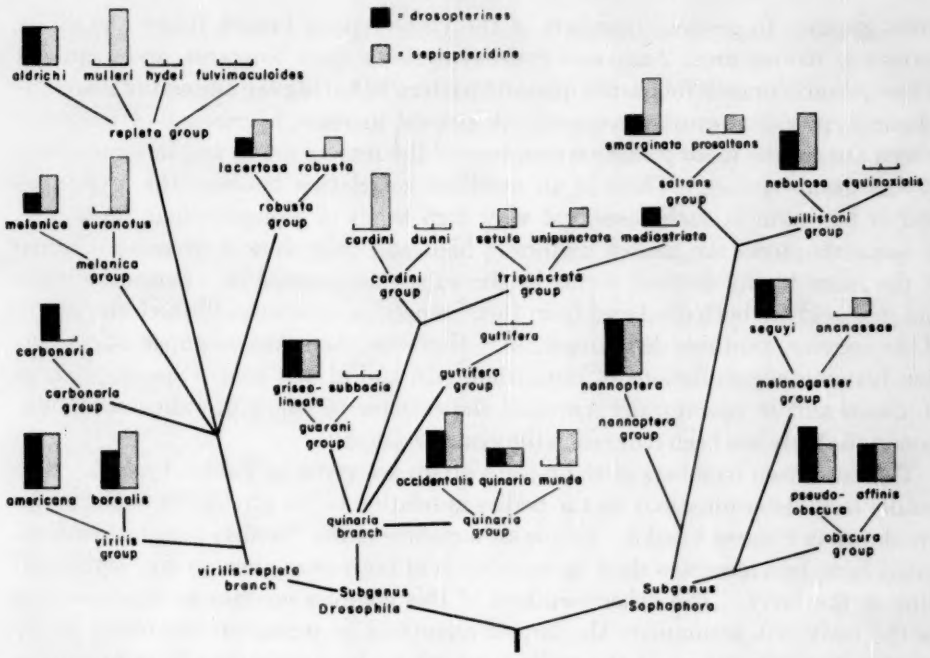

FIG. 2.—Accumulations of drosopterines and sepiapteridine in the bodies of representative species from the genus *Drosophila*.

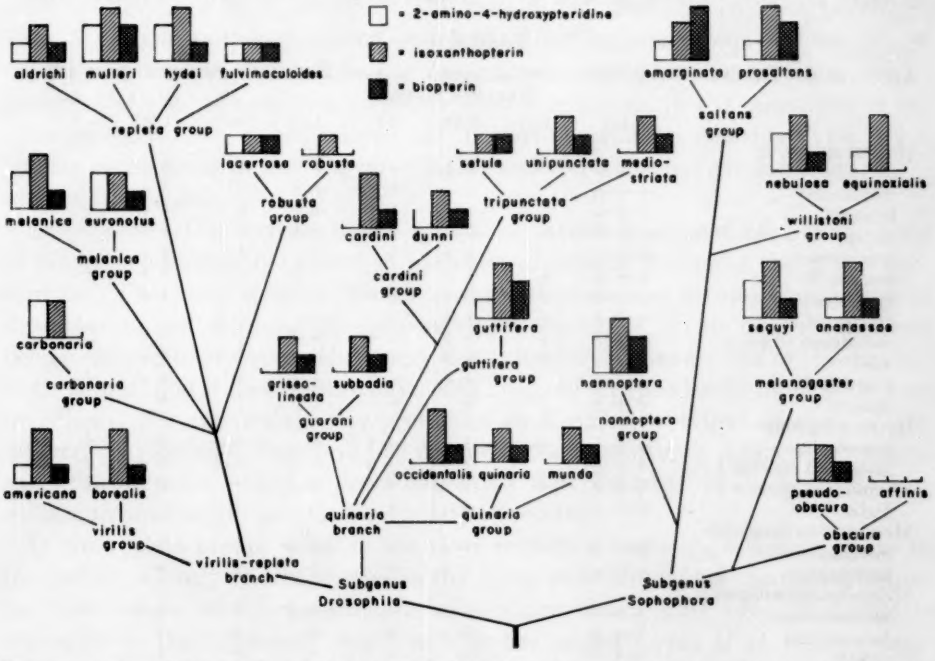

FIG. 3.—Accumulations of 2-amino-4-hydroxypteridine, isoxanthopterin, and biopterin in the bodies of representative species from the genus *Drosophila*.

virilis group. In general, members of the virilis-repleta branch follow the virilis pattern in having more 2-amino-4-hydroxypteridine than biopterin, and members of the quinaria branch follow the quinaria pattern in having the amount of 2-amino-4-hydroxypteridine greatly reduced. A general increase in amount of biopterin is seen among the more primitive members of the repleta group and in some of the saltans group species. There is an excellent correlation between the accumulation of biopterin in such cases and very high levels of sepiapteridine. Amounts of isoxanthopterin are almost uniformly high and only show a decrease in some of the more highly evolved forms in the subgenus *Drosophila*. Isoxanthopterin and uric acid are both produced from their respective substrates through the action of the enzyme, xanthine dehydrogenase. However, there was no apparent correlation between accumulations of isoxanthopterin and of uric acid. Species such as *D. dunni* and *D. equinoxialis* represent the extreme to which pteridine accumulation in the body has been reduced in the genus *Drosophila*.

The data from members of the repleta group are given in Tables 4 and 5. The results from determinations on the bodies in relation to the phylogeny of the group are shown in Figures 4 and 5. Of the six members of the "mulleri complex" investigated here, two members show accumulation of both drosopterines and sepiapteridine in the body. The other members of this complex contain no drosopterines in the body but accumulate the largest quantities of sepiapteridine found in the genus. Other members of the mulleri subgroup also accumulate large quantities of sepiapteridine, but none accumulate it to the extent that the members of the "mulleri complex" do. In the hydei subgroup, none of the species have drosopterines in the body. The sequence from *D. nigrohydei* through the two unnamed species to *D. hydei* shows a graded series from high accumulation of sepiapteridine

TABLE 4

AMOUNTS OF VARIOUS COMPOUNDS ACCUMULATED IN THE HEADS OF SPECIES FROM THE REPLETA GROUP

	DRO	ISO	XAN	SP	AHP	BIO	URC	XIC
Mulleri subgroup								
aldrichi	+++		++	+	+	+	++	
mojavensis	+++		++	++	++		++	+
buzzatii	+++		++	++	++	+	++	+
anceps	+++	+	++	++	++	++	++	
arizonensis	+++		++	+	+	+	++	
hamatofila	+++	+	++	+	++		++	+
longicornis	+++	+	++	+	++		++	
martensis	+++	+	++	+	+	++		
meridiana rioensis	+++	+	++	++	++	++	++	
mulleri	+++	+	++	+	++	++	++	
nigricruria	+++		++	+	++	+	++	+
stalkeri	+++		+	+	+	+	+	
Hydei subgroup								
nigrohydei	+++	+	++	++	+	++	+	+
unnamed species 1	+++		++	++	++	++	++	+
unnamed species 2	+++	+	++	++	++	+	++	+
hydei	+++		+	+		++		
Mercatorum subgroup								
mercatorum	+++		++	+	++	+	++	
paranaensis	+++		++	+	+	+	+	+
Melanopalpa subgroup								
melanopalpa	+++		++	+	++	+	++	+
fulvimacula	+++		++	+	+	+	+	+
repleta	+++		+	++	+	+	+	+
canapalpa	+++		++	++	++	+	++	
fulvimaculoides	+++		++	++	+	++	++	

TABLE 5

AMOUNTS OF VARIOUS COMPOUNDS ACCUMULATED IN THE BODIES OF SPECIES FROM THE REPLETA GROUP

	DRO	ISO	XAN	SP	AHP	BIO	URC
Mulleri subgroup							
aldrichi	++	++	+	++	+	+	+++
mojavensis	++	+++	+	+++	++	++	+++
buzzatii	++	+++	++	+++		+	++
anceps	+	+++	+	+++	+	+	++
arizonensis		+++	++	+++	+	+	++
hamatofila		++	+++	+++	++	++	++
longicornis		+++	++	+++	++	++	++
martensis		+++	++	+++	++	++	
meridiana rionensis		+++	++	+++	+	+++	+
mulleri		+++	++	+++	++	+++	++
nigricruria		++	++	+++	++	++	+
stalkeri		+++	+	++	++	+	+
Hydei subgroup							
nigrohydei		+++	+	+++	+	++	
unnamed species 1		+++	+	++	++	++	++
unnamed species 2		+++	++	++	++	++	+++
hydei		+++			++	+	
Mercatorum subgroup							
mercatorum		+++	++	++	++	++	
paranaensis		+++			+	+	
Melanopalpa subgroup							
melanopalpa		+++		+	++	+	+
fulvimacula		++		++	++	+	
repleta		++		++	+	+	+
canapalpa		++		++	+		++
fulvimaculoides		+			+	+	

to the absence of this compound (see Figure 4). Members of the melanopalpa and mercatorum subgroups contain no drosopterines and have intermediate or very low levels of sepiapteridine. The amounts of the three colorless pteridines (Fig. 5) are generally high among members of the repleta group. There are, in general, high levels of biopterin when amounts of sepiapteridine are high. Some members of the melanopalpa subgroup show a reduction in the quantities of the colorless pteridines. This follows the pattern previously mentioned for other species in the genus in that the more highly evolved forms have reduced quantities of these compounds.

Discussion.—The decrease in the amount of pteridines accumulated in the body of the fly is consistently evident in members of derived groups in the genus *Drosophila*. The most striking change is the reduction and virtual elimination of drosopterine and sepiapteridine deposition in the testes. This decrease has not been restricted to any one evolutionary line. Rather, it has occurred in all branches of the genus, and it has, presumably, been initiated independently in each of these branches. The elimination has proceeded to a different extent in each of the different phylogenetic lines and has produced the spectrum of differences we now see. Furthermore, there is good indication that different methods have been utilized to produce phenotypically similar end results.

In the repleta group, a few of the more primitive forms show drosopterines in the testes. Closely related species in the group have eliminated the drosopterines but accumulate very large amounts of sepiapteridine. This type of change is analogous to the "terminal" block seen in the mutant sepia of *D. melanogaster*. In the more derived forms from the repleta group there is a consistent reduction in

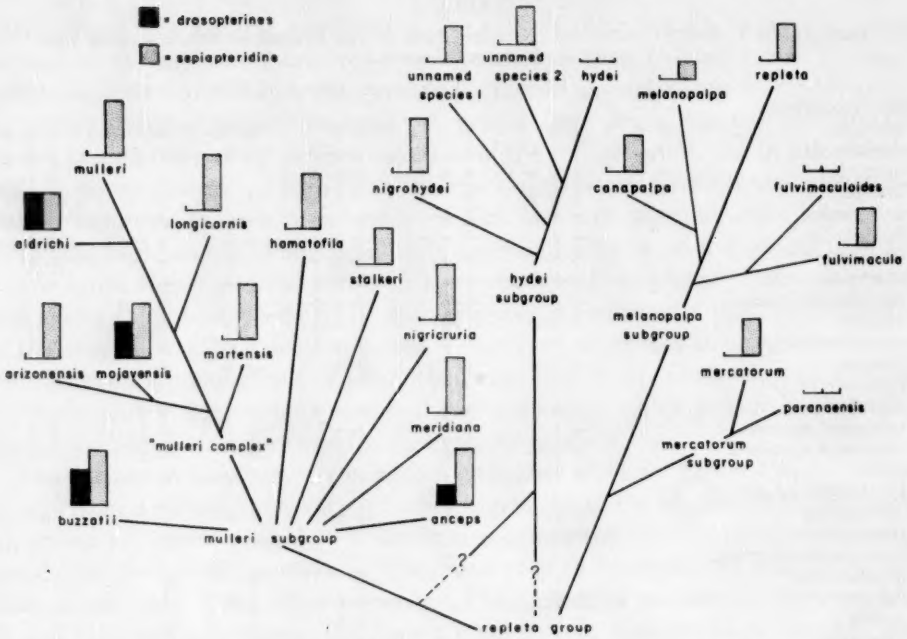

FIG. 4.—Accumulations of drosopterines and sepiapteridine in the bodies of species from the repleta group of the subgenus *Drosophila*.

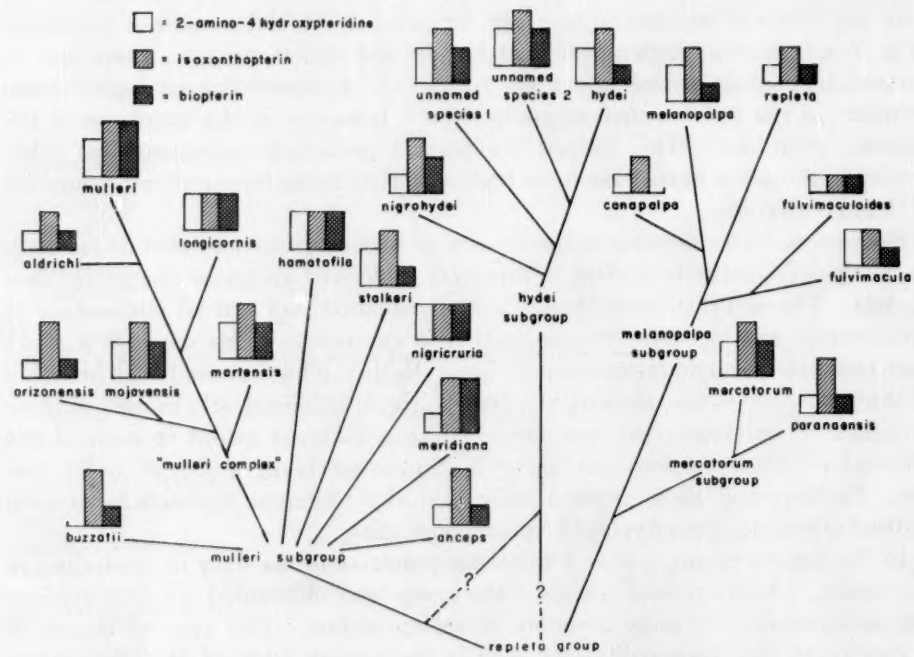

FIG. 5.—Accumulations of 2-amino-4-hydroxypteridine, isoxanthopterin, and biopterin in the bodies of species from the repleta group of the subgenus *Drosophila*.

the amount of sepiapteridine and finally an almost complete absence of accumulation of this compound. Species in the hydei subgroup of the repleta group give a particularly good example of this sequential reduction in accumulation of sepiapteridine.

In the quinaria branch of the subgenus *Drosophila* the accumulation of drosopterine and sepiapteridine is similar to that seen in the derived forms from the virilis-repleta branch. There is, however, an interesting difference. Many members of this branch still contain traces of drosopterines in the testes. This is true even in some of the more derived forms. It can be inferred that the method of reduction in this branch has been different from that of the virilis-repleta branch. It may have involved a block in reactions earlier in the sequence of pteridine metabolism. The mutant *brown* of *D. melanogaster* accumulates practically no pteridines in the eyes,[5] suggesting a block early in pteridine biosynthesis. The general similarity between the accumulations by the *brown* mutant and by the more derived species from the quinaria branch suggests that similar stages in pteridine metabolism may have been affected in each case.

The results obtained from the subgenus *Sophophora* are somewhat different from those obtained from species of the quinaria branch. There are some resemblances between the Sophophoran species groups and the repleta group, however. All groups in the subgenus *Sophophora* appear to have arisen at a time when the basic genotype of the population led to the accumulation of drosopterines in the body. Subsequent to the separation of the major groups, reduction of pteridine accumulation has proceeded independently in each group. This situation can be contrasted with that in the quinaria branch where each of the successive groups in the phylogenetic sequence appears to have arisen with more reduced accumulation of pteridines than was seen in the group which preceded it in the sequence.

With regard to the compounds, drosopterine and sepiapteridine, evolution within the genus has been convergent. At present, the methods utilized to bring about this result can be interpreted as partial or complete metabolic blocks at different steps in the biosynthetic sequence, or as the shunting of intermediary metabolites into a different metabolic sequence. Further, the evolution of this system has involved an organ specific divergence, since the eye at all times has retained the ability to accumulate these pigments. It is not known where the synthesis of these pigments occurs in the organism. The accumulation of sepiapteridine in the eye of the mutant *sepia* and in the testes of other forms strongly suggests that it is produced in the organ in which it accumulates. If the colored pteridines are produced elsewhere in the body and are simply accumulated by the eyes and testes, the results here could be caused by changes in accumulation mechanisms rather than by changes in the biosynthetic pathway.

The original observation that suggested that biopterin might be in the biosynthetic pathway of drosopterine formation (see Fig. 1) came from its accumulation, along with sepiapteridine, when drosopterine accumulation is reduced in the eye of the mutants *maroon-like* and *rosy*^{2} of *D. melanogaster*. An analogous accumulation of biopterin occurs in some forms where sepiapteridine is accumulated, e.g., *D. mulleri*, *D. nannoptera*, *D. emarginata*. This again suggests a precursor relationship of this material.

High levels of 2-amino-4-hydroxypteridine are found in species from the sub-

genus *Sophophora* and from the virilis-repleta branch of the subgenus *Drosophila*. The accumulation of this compound is reduced only in the more derived forms from either of these major groups. It is uniformly low in all species from the quinaria branch of the subgenus *Drosophila*. Isoxanthopterin accumulation is high in all groups except in those where pteridine accumulation is at its lowest level. The two compounds, 2-amino-4-hydroxypteridine and isoxanthopterin, are metabolically related through the enzyme xanthine dehydrogenase. This enzyme has been studied extensively in *D. melanogaster*.[18, 19] Reliable methods for assaying the enzyme have been established and it is possible to determine the level of enzyme activity in different genotypes of this species.[8] The data from the genus suggest that the efficiency of this reaction is greater in members of the quinaria branch than in other members of the genus. Such a possibility could be investigated by present comparative enzyme methods.

The mechanisms responsible for reduction in levels of accumulation of the noncolored pteridines may have been different from those which effected the changes in drosopterine and sepiapteridine accumulation. One possibility is that increased excretion of pteridines has resulted in lowered accumulations within the body. Increased excretion of pteridines occurs in some eye-color mutants of *D. melanogaster*. Hadorn[20] has reported that some mutants lacking the normal pteridine complement excrete large quantities of pteridines shortly after emergence from the puparium. The chemical and physiological similarities of uric acid and isoxanthopterin make the latter compound a logical choice as an end product of pteridine metabolism. This compound has been regarded as the end product of a "shunt" pathway by Robertson and Forrest.[21] Increased activity of such a shunt pathway, perhaps coupled with increased excretion of the end product, may have produced the picture of pteridine accumulation seen in the species from the quinaria branch. In all species investigated here, isoxanthopterin has been the last compound to remain at relatively high levels of accumulation. This again suggests its role as a final product of pteridine metabolism.

When the species of the genus are considered as a whole, two major types of change in pteridine metabolism in the body are apparent. One of these types is best shown by species in the repleta group. Here, the evolutionary sequence seems to have been from species with a primitive, or virilis-type, pteridine phenotype through a type with drosopterines completely eliminated and sepiapteridine accumulated in large amounts, to a series of species in which sepiapteridine accumulation has been sequentially reduced. The second type is that shown by species from the quinaria branch. Here, the first evolutionary step seems to have been a partial metabolic block early in the sequence of metabolic transformations. This has led to a great reduction in accumulation of all pteridines, with the exception of isoxanthopterin. Increased activity of a "shunt" pathway might also have produced this pattern of pteridine accumulation.

In general, members of species groups from the subgenus *Sophophora* resemble those from the repleta group more closely than those from the quinaria branch. Since the relationships of species within Sophophoran groups are not well established, it is not possible to establish for these species a sequence of change such as was done for species in the repleta group. It is likewise not yet possible to interpret the results from other groups in the subgenus *Drosophila*.

Summary.—A large number of species from the genus *Drosophila* have been investigated to determine the extent to which each accumulates known pteridines. Results were correlated with taxonomically established phylogenetic relationships to relate the types of change which have occurred in pteridine accumulation during the evolution of the genus. Representatives from primitive groups accumulate moderate quantities of pteridines, including the red eye pigments (drosopterines), in the testes. The amounts of these compounds accumulated in the testes have been greatly reduced in members of derived groups from each of the four major evolutionary lines studied. This type of change appears to have occurred independently in each of these lines, although phenotypically similar end results have been achieved in most cases. Evidence suggests that at least two mechanisms have been utilized to produce these results. In one case, the first evolutionary step appears to have involved a "terminal" metabolic block resulting in complete elimination of drosopterine accumulation and in very great accumulation of sepiapteridine and biopterin. In the other case, the first step appears to have involved a partial metabolic block early in the sequence of pteridine biosynthesis. This has resulted in a great reduction in amounts of most pteridines accumulated, but drosopterines still accumulate in trace amounts. This last type may have involved increased activity of a shunt pathway, perhaps coupled with increased excretion of pteridines.

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*HYBRIDIZATION BETWEEN LYCOPERSICON ESCULENTUM AND SOLANUM PENNELLII: PHYLOGENETIC AND CYTOGENETIC SIGNIFICANCE**

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Those species of the immense genus Solanum that bear the closest morphological resemblance to species of Lycopersicon are classified in Subgenus Pachystemon, Section Tuberarium, Subsection Hyperbasarthrum, Series Juglandifolia.[1] One species of this series—*S. lycopersicoides* Dun.—was successfully hybridized with *L. esculentum* Mill., the garden tomato, in 1951.[2] Although this hybrid is instructive in respect to its viability and chromosomal behavior, its complete auto-sterility and cross-incompatibility between all combinations of the diploid and tetraploid forms of the hybrid and of the tomato preclude further cytogenetic studies that might have shed light on the phylogenetic relations between the two genera. Attempts to hybridize several accessions of *S. ochranthum* H.B.K., another member of the Juglandifolia series, with tomato species have failed. Opportunities for testing other members of this series were therefore eagerly awaited. Interest in this subject has been renewed by the naming of a new species—*S. pennellii*—by Correll in 1958.[3] Initial results on the hybridization of this species and *L. esculentum* form the substance of this article.

Solanum pennellii is known from several stations in coastal Peru, and material for the present study was kindly furnished by Dr. Correll from a single collection (PI 246502) made between Chala and Atico in the Department of Arequipa, Peru. As this accession grew in our cultures it agreed in all morphological respects with Correll's description. It lacks the sterile anther tips, which constitute the key generic character of the genus Lycopersicon. Noteworthy also are the basal articulation of the pedicels (anomalous for subsection Hyperbasarthrum) and the joining of anthers to form a tube (as in Lycopersicon and very few species of Solanum). In general aspect *S. pennellii* shows closer morphological resemblance to the tomatoes than to the nightshades. In common with all known species of Lycopersicon and of the Juglandifolia series of Solanum, it has 12 pairs of chromosomes.

Results.—Without exception all plants of *S. pennellii* set fruit freely after controlled self-pollination, and no barriers to crosses within the species were encountered. Such behavior is exceptional because all the closely related species of Solanum heretofore tested and most wild species of Lycopersicon are known to be self-incompatible; furthermore, within closely related groups, self-incompatibility is generally associated with flowers having such entomophilous traits as the follow-

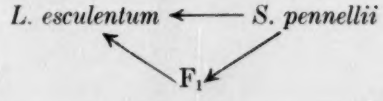
ing found in *S. pennellii:* large, showy, well-displayed flowers, copious pollen, and exserted stigmas. It must be borne in mind, however, that our experience has been limited to a single collection, which possibly might not be typical of the species as a whole.

Even more unexpected than self-fertility is our finding of moderate compatibility between *L. esculentum* and the new nightshade. Crosses utilizing the former as female parent set fruit with a variable number of viable seeds; the reciprocal cross yields either no fruit or tiny abortive fruit. Germination of the hybrid seeds is retarded, but otherwise the hybrid seedlings grow without special care. The hybridization is thus effected nearly as readily as intraspecific crosses in either parent and with vastly greater ease than in most crosses among tomato species[4] and between them and *S. lycopersicoides.*[2] This unusual compatibility situation also contrasts sharply with the barrier found in all crosses attempted thus far between *S. pennellii* and other nightshade and tomato species.[5]

The appearance of parental traits in all the offspring dispels any doubts concerning their hybrid origin. For some 30 well defined character differences between the parental species, the usual dominance of the wild parent is exhibited in 11; for six the tomato traits were dominant; and for 13 other characters the F_1 was strictly intermediate. For all known *esculentum* species hybrids this proportion of cultigen traits that are dominant or intermediate in the F_1 hybrids is unusually large.

A third extraordinary situation is the high level of fertility of the F_1 hybrids. Whereas the *esculentum-lycopersicoides* hybrids proved totally sterile, the ones presently reported have 75 per cent stainable pollen; their seed yields after self-pollination are about 30 per cent of parental levels; and about 40 per cent of the seeds germinate. Little degeneration has been encountered in the F_2 generation. In these respects also the hybrids present far less of a sterility barrier than most hybrids between tomato species.

The results of hybridizations in all possible combinations between parents and F_1 hybrids are summarized in the following figure (arrows designate the only directions of successful crosses):



In the incompatible crosses either no fruits or small abortive seedless fruits are formed. By applying Martin's fluorescence technique[6] it has been possible to ascertain that in the cross of *S. pennellii* ♀ × *L. esculentum* ♂ the majority of pollen grains fail to germinate, but a few produce tubes that traverse the entire length of the style. For the unsuccessful cross of F_1 ♀ × *L. esculentum* ♂ most grains germinate and tubes grow at variable rates, some reaching the base of the style in 24 hours.

The pattern of unilateral compatibilities outlined above corresponds exactly with that found in hybrids between *L. esculentum* and self-incompatible tomato species.[7] The agreement suggests that *L. esculentum* might be a primary self-fertile species and *S. pennellii* secondarily so according to Lewis' proposal[8] of derivation of the latter from a self-incompatible species by mutation of an *S* allele. A difficulty

encountered in this interpretation is the difference found in the nature of the incompatibility response: non-germination in the present example and retarded pollen tube growth in self-pollinations of the related self-incompatible species. Another problem is presented by the activity of other genes, for evidence has been found for the control of the unilateral barrier between tomato species not only by *S* alleles but also by independent genes determining pollen behavior in a sporophytic fashion.[7] Possibly the latter genes might have become fixed to reinforce the barrier to crossing that was imposed by the *S*-allele system.

Meiotic chromosomes in pollen mother-cells of the hybrids behave in a fashion commensurate with the high reproductive fertility. The only deviations detected from normal comportment were the formation of a quadrivalent or secondary association between two bivalents in some 20 per cent of the diakinetic figures and lagging univalents in about five per cent of the metaphase and anaphase figures. Four bivalents are visibly heteromorphic for size of the proximal chromatic region, but these differences do not prevent normal synapsis and chiasma formation.

Tetraploid shoots were readily generated by colchicine treatment of axillary buds of the F_1 hybrids. Gametic fertility of the tetraploids does not quite equal that of the diploids (65 per cent stainable pollen), but zygotic fertility as measured by seed yields is considerably higher. Meiotic chromosomes form various numbers of multivalents with an average of 3.0 per cell. This chromosome behavior, being considerably more orderly and the reproductive fertility much higher than in tomato autoploids, suggests some degree of chromosomal differentiation between the two species. The tetraploid hybrids are therefore segmental alloploids inasmuch as they represent a condition intermediate between the extremes of strict autoploidy and alloploidy.[9]

Discussion.—Numerous intriguing problems are posed by the acquisition of *S. pennellii* and by the hybridization studies. The problem of systematics, although not the most important basically, is the most immediate one especially because it concerns the taxonomy of an economic crop of worldwide importance. From the results presented above, it is clear that the genetic affinities of *S. pennellii* are closer to Lycopersicon than to Solanum, yet this species possesses the key anther traits of a Solanum. It must be admitted that the evidence of genetic relationships would be greatly strengthened by a study of hybrids between *S. pennellii* and other species of Solanum. Yet, even if such hybrids could be produced, it is highly doubtful whether they would prove as fertile as the existing hybrids with Lycopersicon. The previously described hybrid between *L. esculentum* and *S. lycopersicoides* is completely sterile, and the sterility is known to have both genic and chromosomal components.[2] Now if we take into account the high chromosome homology and remarkable genetic compatibility between *L. esculentum* and *S. pennellii*, it appears extremely unlikely that a close genetic relationship could exist between *S. pennellii* and *S. lycopersicoides*. Morphological comparisons lead to a similar prediction, for the two Solanum species differ from each other at least to the same extent that either differs from *L. esculentum*.

The systematic problem cannot be fully treated in this article, and additional studies are needed before a firm conclusion can be reached. The available facts are adequate, however, to reveal that revision is needed, not only for the status of *S. pennellii* but also for the concept of the genus Lycopersicon. Uncertainty about

the proper classification of this species was also expressed recently by Correll[10] after reconsideration of certain morphological characters. The new light thus shed on the relations between Solanum and Lycopersicon casts additional doubts on the distinctness of the latter.

Another consideration that is underscored by the present discoveries deals with the unique phylogenetic position of *L. esculentum*. It is now apparent that this species (and probably also the closely related *L. pimpinellifolium*) behaves cytogenetically as a common denominator among the tomato species and closely related nightshade species: it is the only species to which all other tomato species can be hybridized, and it is also the only tomato species which has yielded viable hybrids with either *S. lycopersicoides* or *S. pennellii*. The cultivated tomato will furthermore produce hybrids of complete or moderate fertility with the following remarkably diverse entities: *L. pimpinellifolium* (Jusl.) Mill.,[11] *L. hirsutum* f. *glabratum* C. H. Mull.,[12] *L. esculentum* var. *minor* Hook. (*L. cheesmanii* f. *minor*),[13] and *S. pennellii*.

It is also noteworthy that all members of this intercompatible complex are self-compatible whereas all other known entities in Lycopersicon and the Solanum series Juglandifolia are strictly self-incompatible. This example is therefore exceptional to the general situation among higher plants, in which barriers to hybridization seem to be more rapidly acquired by autogamous species than allogamous ones.

The remarkable cross-compatibility within this complex might be explained as the result of differentiation from a common ancestor or of hybridization and introgression between intercompatible ancestors. As to the former, the highly diverse entities of this group might have diverged by mutation, segregation, and the formation of new combinations from an ancestral form, probably no longer in existence. According to this hypothesis cross-fertility was preserved presumably because it remained selectively neutral. The self-fertility prevailing in this group would have greatly abetted differentiation by enforcing rapid segregation and gene fixation. Differentiation might have also been greatly expedited by geographical isolation. It was pointed out previously[13] that these two isolation mechanisms might have played important roles in the evolution of *L. esculentum* var. *minor* on the Galápagos Islands. On the other hand, it is tempting to suppose that the intercompatibility and part of the morphological diversity were derived from hybridization. It is easy to conceive, for example, that phenotypes of the primitive forms of *L. esculentum*—var. *cerasiforme* and *minor*—could be approximated by various combinations of parental traits of *L. pimpinellifolium* and *L. hirsutum*. Since the available data do not discriminate between these two hypotheses, the phylogenetic meaning of this situation is not clear at the present time.

One of the most fascinating aspects opened by the acquisition of *S. pennellii* is the opportunity for cytogenetic analysis of the hybrids with *L. esculentum* and their derivatives. As indicated above, a preliminary comparison of the two species revealed no less than 30 distinguishing traits. The inheritance of these traits *per se* will be of interest in revealing the degree of genetic complexity in the determination of characters that have been considered valid for separating genera, species, and other systematic categories. The favorable pachytene stage in these materials should provide a unique opportunity for studies of evolutionary changes in chromosome structure.

Especially welcome will be the vast new store of germplasm that can be released for transfer to *L. esculentum* by means of standard backcross procedures. A few of the aspects that should afford profitable investigation might be mentioned. The pattern of unilateral compatibility relations outlined above should be analyzed genetically and compared with that of *L. esculentum* and the self-incompatible species. The behavior of *pennellii* chromosome segments when transferred into the genotypic milieu of *L. esculentum* should be investigated. Finally, the enrichment of the gene pool of the latter species for breeding purposes and formal genetics alone would justify continued study.

Summary.—*Lycopersicon esculentum* can be hybridized with *Solanum pennellii* to yield viable hybrids without the use of special aids, although it has been possible to make the cross only with the former as female parent. For nearly half of the many morphological character differences between the two species the F_1 hybrids are intermediate; for about one-fifth the tomato traits are dominant; for the remainder the nightshade traits are dominant. In common with both parents, the hybrids have 12 pairs of chromosomes. Despite their heteromorphy, the chromosomes of both parents pair and otherwise function in meiosis of the hybrids with nearly normal regularity. The net reproductive fertility, as measured by the number of F_2 progeny produced after self-pollination of the hybrids, is about 12 per cent of normal. Chromosomes of induced tetraploid hybrids form multivalents, but at a frequency lower than that of autoploid tomatoes. Doubling the chromosome number results in a considerably higher seed yield of the hybrids—a fact that implies appreciable chromosomal differentiation between the parental species. The anomalous genetic affinity between the parents, despite their possessing the key taxonomic characters that separate the two genera, casts doubt on the validity of the genus Lycopersicon. The discovery of *S. pennellii* and its compatibility with *L. esculentum* opens a large field of inquiry into compatibility relations, introgression, and tomato breeding and genetics.

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MITOTIC REPLICATION OF DEOXYRIBONUCLEIC ACID IN CHLAMYDOMONAS REINHARDI

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One of the central problems in molecular biology is to elucidate the replication mechanism of deoxyribonucleic acid (DNA) and its possible implication in genetic recombination. Of the several ingenious techniques that have been developed for the investigation of this problem,[1, 2, 3] the density gradient centrifugation technique developed by Meselson, Stahl, and Vinograd[4] provides the most promising method, and has already furnished the clearest picture of DNA replication in *Escherichia coli*.[5]

Here we have initiated a series of experiments in which the density gradient centrifugation technique has been used to investigate the mechanism of DNA replication in the chromosomes of an organism with clearly defined *mitosis* and *meiosis*. The unicellular green alga, *Chlamydomonas reinhardi*, was chosen as the experimental material because it possesses a number of advantageous characteristics. The alga can be grown easily in a minimal medium, the life cycle is extremely simple and yet it includes the characteristic features of higher organisms: vegetative growth, fertilization, and meiosis which can be readily controlled.[6] The chromosomes of *C. reinhardi* are well defined,[7, 8] and it has been established that the genetic segregation follows the classical meiotic pattern.[9, 10, 7] The present paper reports the mode of mitotic replication of *C. reinhardi* DNA. It is planned to report in a later paper the investigation of meiotic replication.

Isolation and Properties of DNA of C. reinhardi.—A wild-type, mating type plus strain of *C. reinhardi* was grown in a high salt minimal medium (Table 1) at 25°C

TABLE 1

Composition of Minimal Liquid Media

	Minimal* Medium	High Salt Minimal Medium
NH_4Cl, (gm)	0.05	0.50
$MgSO_4 \cdot 7H_2O$, (gm)	0.02	0.02
$CaCl_2 \cdot 2H_2O$, (gm)	0.01	0.01
K_2HPO_4, (gm)	0.72	1.44
KH_2PO_4, (gm)	0.36	0.72
Trace element solution,† (ml)	1	1
Distilled water, (liter)	1	1

* The minimal medium is the same as that of reference 10, except that NH_4Cl was substituted in place of NH_4NO_3.
† Hutner *et al.*[11]

in two-liter Erlenmeyer flasks. The cultures were aerated with five per cent carbon dioxide in air and stirred constantly by a magnetic stirrer. Illumination was from two sides and from above by Ken-Rad 30 watt daylight fluorescent lamps, which gave a light intensity of about 600 foot candles in the center.[12] Cell samples were withdrawn from a side siphon without opening the culture flask. When a cell concentration of 2×10^7 per ml was reached, the cells were centrifuged and washed once with a saline citrate solution (0.15 *M* NaCl plus 0.015 *M* sodium citrate, pH 7)

and suspended in 4 ml of the same solution for every 10^{10} cells. The cells were lysed by addition of sodium dodecyl sulfate (final concentration three per cent), and the DNA was purified by a chloroform-octanol method.[13] DNA preparations which contained three per cent protein by Folin-Ciocalteu method[14] have been obtained.

Both the purified DNA as well as DNA from the direct lysate were centrifuged in a 7.7 molal CsCl solution with calf thymus DNA as a density reference. It was found that the effective density of *C. reinhardi* DNA was, for both preparations, unusually high (1.728)[15] compared with that of DNA from phage, calf thymus,

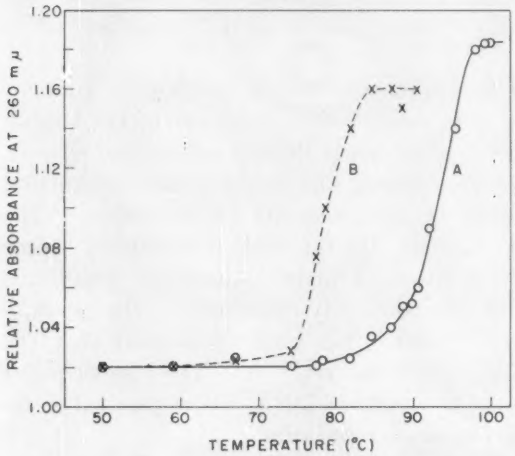


FIG. 1.—Hyperchromic shift of *C. reinhardi* DNA as a function of the temperature. Curve A: purified DNA of *C. reinhardi* was dissolved in the saline citrate solution (0.15 *M* NaCl plus 0.015 *M* sodium citrate, pH 7). Curve B: the same DNA in the saline citrate solution was dialyzed against a diluted saline citrate solution (0.015 *M* NaCl plus 0.0015 *M* sodium citrate, pH 7) at 3°C for 24 hours. The solution outside the dialysis tubing was changed three times and constantly stirred by a magnetic stirrer. The two DNA solutions in cuvettes were heated in the cuvette chamber of a Beckman DU spectrophotometer equipped with heating system,[16] and the optical densities at 260 mμ measured at various temperatures. The initial optical densities of the two solutions were 0.372 (A) and 0.223 (B).

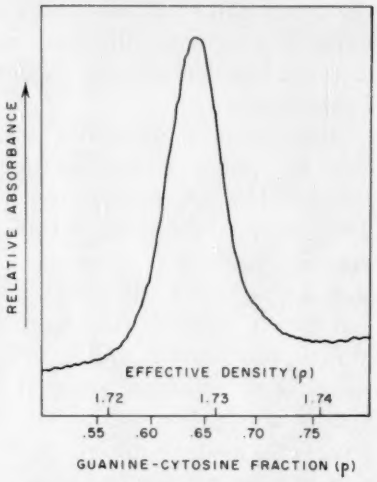


FIG. 2.—Distribution of purified *C. reinhardi* DNA in density gradient field. The ultraviolet absorption photograph of the equilibrated DNA band after 50 hours of centrifugation at 44,770 rpm (at 25°C) was traced by a Joyce-Loebl double beam microdensitometer. The density gradient at the peak position is 0.124 gm cm^{-3}. The guanine-cytosine scale was obtained from the equation $\rho = 0.103\ p + 1.662$[18], where ρ is the effective density and p is the molar fractional content of guanine and cytosine. The peak density is 1.728 and the apparent molecular weight calculated from the band profile is five million.

salmon sperm, and *E. coli* which range in density from 1.70 to 1.71. The fact that the purified DNA of *C. reinhardi* is not denatured is evident from a sharp hyperchromic shift obtained by heating it in the saline citrate solution (Fig. 1). The extremely high denaturation temperature of the DNA suggested a high guanine-cytosine content,[17] and, consequently, the possibility of a general correlation between the base composition and effective density of DNA was inferred. The latter possibility was explored by centrifuging DNA samples from various sources, and it was found that the effective density did increase linearly with the increase of the guanine-cytosine content.[18] According to this relationship *C. reinhardi* DNA

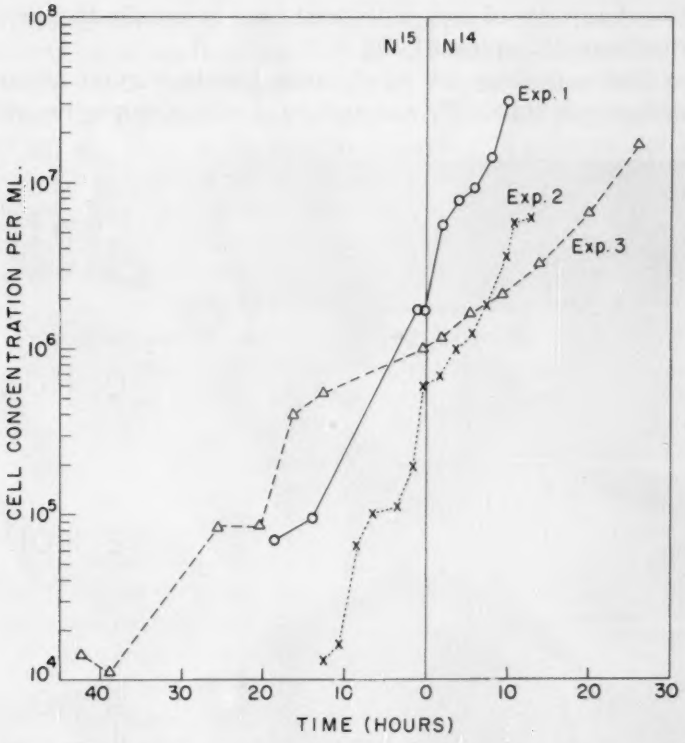


FIG. 3.—The growth curve of *C. reinhardi* in transfer experiments. The cell number was determined with the aid of a hemocytometer. The values on the ordinates have been corrected for the dilution by withdrawals of samples and addition of supplementing medium. At zero time the N^{15} medium was diluted with N^{14} supplementing medium. The medium used for Experiment 3 is different from that of the other two experiments in that the calcium content was reduced to half and 2 gm of sodium acetate per liter was added. The average generation time is about three hours in Experiments 1 and 2 and seven hours in Experiment 3. The error in the cell counting is usually within ten per cent. The stepwise growth curve comes from a partial synchrony of cell divisions and the characteristic growth habit of *C. reinhardi* in liquid culture (see Figs. 6 and 7).

should contain about 65 per cent guanine-cytosine.[19] A tracing of a band profile of the purified DNA is shown in Figure 2. Upon heating the DNA in the CsCl solution for 15 minutes at 100°C the effective density increases by 0.015 gm cm^{-3}. Preliminary results on other physical properties show that the sedimentation coefficient ($s_{20,w}$) of the purified *C. reinhardi* DNA in a solution of 0.15 *M* in NaCl and 0.015 *M* in sodium citrate at pH 7 is 23.4S and according to an empirical equation given in reference 20 corresponds to a weight average molecular weight of nine million. The apparent number average molecular weight calculated from the DNA band profile of density gradient centrifugation is five million. The discrepancy comes most likely from a reflection of the density heterogeneity which exists in the DNA sample and the difference between weight- and number-average molecular weights.[21]

Transfer Experiment.—Three transfer experiments from a nitrogen-15 (N^{15}) medium to a nitrogen-14 (N^{14}) medium have been made under slightly different con-

ditions. Since the results of each experiment were essentially the same, only one will be described here (Experiment 1, Fig. 3).

A two-liter flask containing 500 ml of minimal medium whose nitrogen was replaced by its heavy isotope, N^{15}, was inoculated with a suspension of fresh cells

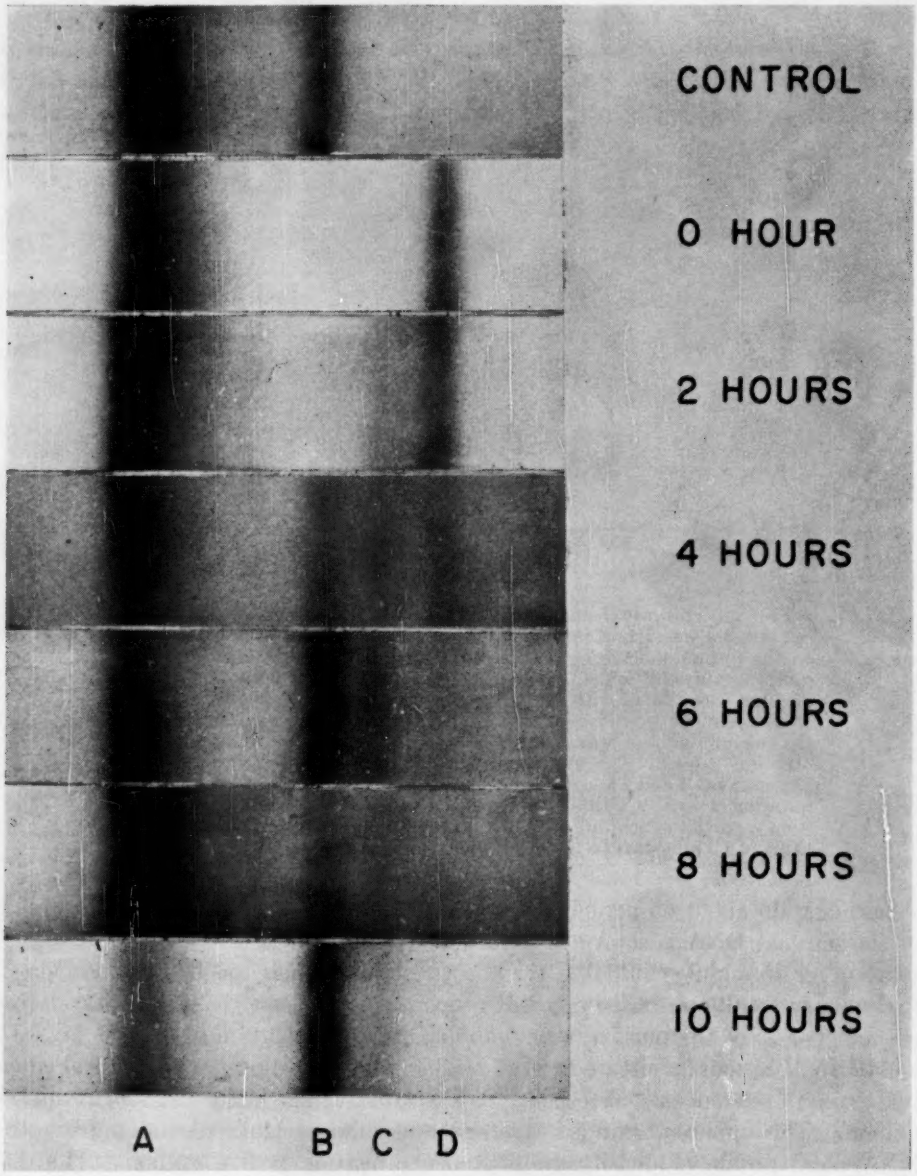


FIG. 4.—Ultraviolet absorption photographs of DNA bands of *C. reinhardi* lysates in density gradient field at various times after the dilution of N^{15}-culture by an excess amount of N^{14} substrates. The rotor speed was 44,770 rpm and the duration 20 hours at 25°C. Band A is calf thymus DNA added as a reference which enables an exact comparison with the other bands. In the first picture native DNA is shown as the control. The generation time is approximately three hours. Densities of the three *C. reinhardi* bands, non-labeled (B), half-labeled (C), and fully-labeled (D), are respectively 1.728, 1.735, and 1.742 at their peaks.

which had been grown on a minimal agar medium (two per cent agar). The initial concentration in the flask was 7×10^4 cells per ml. Samples of about 5 ml were withdrawn occasionally for making cell counts to determine the rate of growth. When the cell concentration reached 1.7×10^6 per ml, the first large aliquot (100 ml) was taken as the zero time sample. In order to replace N^{15} with N^{14}, a supplementing medium which contained ten times as much NH_4Cl as the minimal medium plus each of the four bases, adenine, thymine, guanine, and cytosine at concentration of 10 mg per liter was added to the culture bringing the total volume of the medium to 1.4 liters. Here the atomic dilution of N^{15} by N^{14} was 29 times. A sample of about 100 ml was taken every two hours and the same amount of the supplementing medium was added back to the flask. The growth curve throughout the transfer experiment is given in Figure 3. The cells of each sample were concentrated to about 5×10^8 per ml in a saline citrate solution (0.15 *M* NaCl and 0.015 *M* sodium citrate) and lysed with three per cent sodium dodecyl sulfate. An aliquot of the lysate (0.1 ml to 0.2 ml) was mixed in 0.8 ml of a nine molal CsCl solution. An appropriate amount of water was added to obtain desired density. Determination of the sample density was made by measuring the refractive index of the mixture of lysate and CsCl solution.[22] Following the addition of sodium dodecyl sulfate the protein and practically all pigments float to the surface of the mixture after it stands for a short time. The clear phase of the mixture is taken up by a syringe and put into the cell of the analytical ultracentrifuge. The density gradient was calculated for each experimental condition according to the procedure described by Meselson.[23] Ultraviolet absorption pictures of almost completely equilibrated DNA bands (after 20 hr at 44,770 rpm) of samples taken at various times after the transfer are shown in Figure 4, and the relative amount of DNA in each band at different times as estimated from tracings of the ultraviolet absorption photographs is shown in Figure 5. Figures 4 and 5 are the same experiments and are typical of the results obtained. There are three distinct kinds of molecules: heavy, intermediate, and light with the corresponding densities of 1.742, 1.735, and 1.728, respectively. The fair separation of these three bands indicates the absence of an appreciable pool of N^{15} DNA-precursors. The zero time picture shows that 95 per cent of DNA is heavy, indicating that the replacement of N^{14} with N^{15} is satisfactory. The succeeding pictures show that the transition of the bands is from heavy to intermediate then to light. It is clear that after two hours' incubation in N^{14}-medium (Fig. 4) some heavy DNA trans-

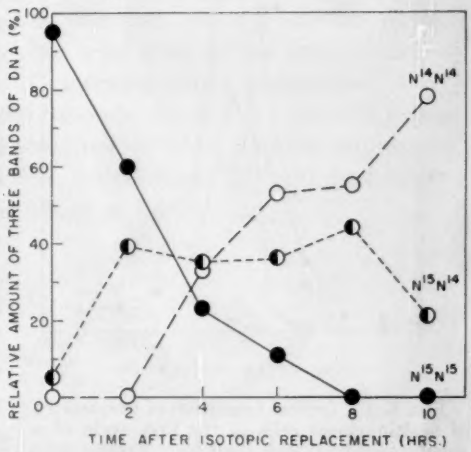


FIG. 5.—Transition of relative amount of fully-labeled ($N^{15}N^{15}$), half-labeled ($N^{15}N^{14}$), and non-labeled ($N^{14}N^{14}$) DNA after the isotope replacement. The relative amounts of the three kinds of DNA were measured from microdensitometer tracings of the ultraviolet absorption photographs whose positives are shown in Figure 4.

forms to intermediate DNA. The light DNA has not yet appeared at this time thus excluding the possibility of the total conservation of the old molecule in DNA duplication. The situation is exactly analogous to that of *E. coli*.[5] The transition of the bands, however, has a special feature, that is, there is a period at four and six hours after the transfer when all three bands are apparent. In *E. coli*, however, only the intermediate band is present at one generation after the isotope replacement. The possible cause of the difference in the transition of the bands in *C. reinhardi* and *E. coli* may lie in different characteristics of their growth, particularly with regard to nuclear division. Accordingly, the growth habit of *C. reinhardi* was analyzed in synchronized liquid cultures. The synchronized cultures were obtained by inoculating liquid minimal medium with fresh cells which had been grown under the light for two to three days on a minimal agar medium supplemented with 4 gm of yeast extract and 2 gm of sodium acetate per liter. The liquid culture was then incubated under the same conditions of light, temperature, and aeration as before. Samples of about 10 ml were withdrawn at various times and the cells immediately chilled to stop further growth. Cell counts and morphological observations were made after the cells had been immobilized with a minute amount of formaldehyde. The chilled cells were also taken to prepare slides for nuclear staining with azure-A.[24]

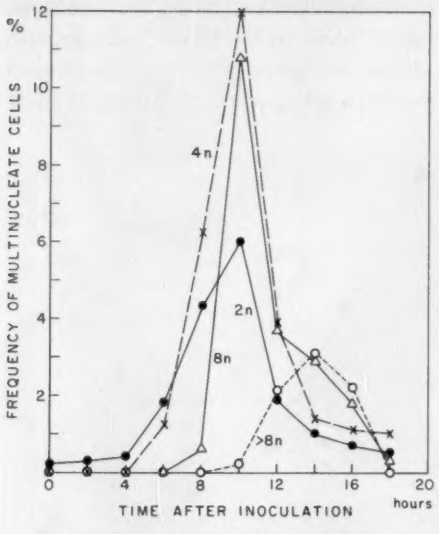


FIG. 6.—A typical transition of frequencies of multinucleate cells in the first cycle of a synchronized liquid culture. Fresh cells from two-day-old yeast agar plates were inoculated in a minimal liquid medium with the initial concentration of 6.4×10^4 cells per ml. For the conditions of culture, see the text. A drop of concentrated cell suspension was smeared on a slide which had been spread with a small amount of egg albumin. The fixation and staining of the preparation were made according to DeLamater.[24] In the figure, 2n, 4n, and 8n indicate the number of nuclei per cell, and >8n means the cell contains more than eight nuclei (mostly 16 and 32). Some of eight nucleate cells in the figure are in the C-colony stage (Fig. 5).

From the cytological observations of stained cells it is clear that before cell division occurs the cell undergoes a number of nuclear divisions. The frequency of cells with two, four, and eight nuclei is given in Figure 6. Figure 6 shows that before certain cells initiate a nuclear division, other cells have already undergone more than three nuclear divisions. It is quite conceivable that a similar situation is also true for DNA duplication. Consequently there is a period when both unduplicated DNA (heavy) and duplicated DNA (intermediate and light) are present thus forming the three bands simultaneously.

Observation of immobilized cells confirms this cytological finding. In order to analyze the cell variations in size and shape, intact cells were arbitrarily classified into three types; A-cells which are about 5 μ–6 μ in diameter, B-cells which are one of a group of cells 7 μ–14 μ in diameter, and C-colonies which consist of a group of unseparated cells from a single mother cell. The A-cells are apparently derived

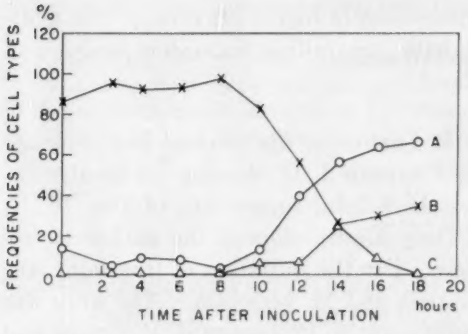
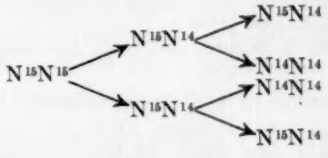


FIG. 7.—A typical frequency transition of cell types of *C. reinhardi* during the first division cycle in a synchronized liquid culture. A: Small cells, 5–6 μ in diameter. B: Large cells: 7–14 μ in diameter. C: Colonies of daughter cells originating from single B-cells. The culture is the same one described in Figure 6.

from separation of the cells of C-colony. The frequency of the occurrence of the different cell types with time is shown in Figure 7. It is clear that following separation of C-colonies the cells gradually increase in volume and then suddenly undergo cell divisions yielding eight or more daughter cells. The over-all picture of the growth pattern of *C. reinhardi* in liquid culture is shown schematically in Figure 8.

This characteristic growth pattern seems to be rather common in Chlamydomonas, since a similar pattern has been observed on agar cultures of *C. moewusii* and *C. eugametos.*[7]

Discussion.—The appearance of the three bands in the N^{15}–N^{14} transfer experiments is best interpreted by the bipartite structure of DNA, and the sequence of the transition among the three states of DNA, fully-labeled ($N^{15}N^{15}$), half-labeled ($N^{15}N^{14}$), and nonlabeled ($N^{14}N^{14}$) is most likely as follows,



This is the model which was proposed by Watson and Crick[25] and described as semi-conservative by Delbrück and Stent.[26]

The transfer experiment on mitosis of *C. reinhardi,* therefore, gives a pattern of DNA replication analogous to that of *E. coli.*[5] This pattern is one in which each continuous half of the DNA subunit is conserved in two daughter molecules of the succeeding generation. The results presented here generalize the finding of

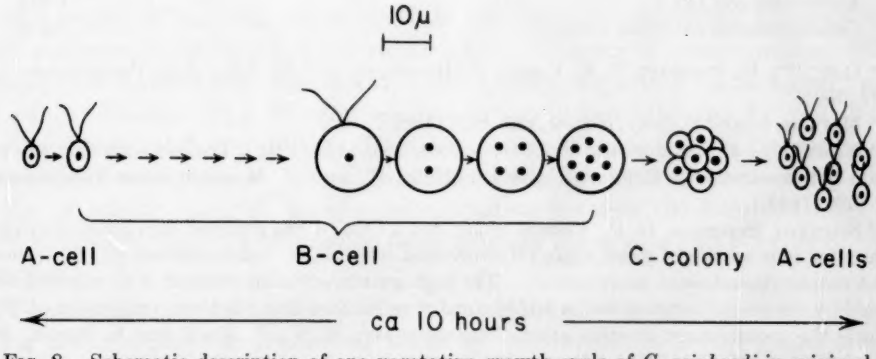


FIG. 8.—Schematic description of one vegetative growth cycle of *C. reinhardi* in minimal liquid culture. The nucleus is shown as a black dot.

Meselson and Stahl for *E. coli* to the chromosomes of higher organisms. In addition the results are formally consistent with the radioautographic analysis of chromosomes by Taylor, Woods, and Hughes.[3]

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Diplococcus pneumoniae DNA	1.703
Calf thymus DNA	1.706
Escherichia coli DNA	1.713
Chlamydomonas reinhardi DNA	1.728

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*GENETIC STRUCTURE OF RECOMBINANT CHROMOSOMES FORMED AFTER MATING IN ESCHERICHIA COLI K12**

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Genetic studies on bacteria and bacteriophage have demonstrated the presence of a linear array of hereditary determinants.[1-3] Experiments on bacterial transformation and phage infection identify DNA (deoxyribonucleic acid) as the carrier of genetic information.[4-5] Replication of DNA has been studied with various organisms.[6-8] Experimental results suggest that DNA molecules are composed of two units, which separate from each other during duplication, to form two daughter molecules, each composed of a parental unit and of a newly synthesized one, and that the units themselves remain intact through succeeding duplications. They are consistent with the model of DNA duplication proposed by Watson and Crick.[9]

In chromosome duplication, the two units of the parental DNA, which are assumed to have equivalent genetic information, transfer the genetic characters of the parent to each of the two daughter chromosomes. However, when two chromosomes recombine, the DNA units of the two parents, which may not be identical, participate in the formation of recombinant chromosome(s). Therefore, the genetic structure of recombinant chromosomes may have characteristics different from those formed in a duplication process not involving recombination. In the present paper the genetic characteristics of recombinant chromosomes formed after mating in *E. coli* K12 are analyzed.

The process of bacterial recombination can be summarized as follows.[10-11] When a culture of *Hfr* cells (male, yielding a high frequency of recombination) and a culture of F^- cells (female) are mixed, some *Hfr* cells pair with F^- cells, forming an intercellular bridge. The *Hfr* chromosome is introduced into the F^- cell unidirectionally through the bridge. If in the course of transfer the chromosome breaks, the F^- cell receives only a fragment of the *Hfr* chromosome. After the transfer of genetic material, genetic recombination between the fragment of *Hfr* chromosome and the F^- chromosome takes place in the zygote and is followed by segregation.

Let us consider several models of the recombinant chromosome(s) formed after mating and examine the predictions made by each model as to the patterns of segregation to be observed in the descendants of a zygote. A bacterial chromosome

is assumed to be composed of two units with equivalent genetic information. The units separate during duplication, each unit going to one of the two daughter chromosomes. If recombination is to occur, at least one of the single units must contain information from both parents. Such a recombinant unit could be formed either by the direct incorporation of part of the *Hfr* fragment into an F^- chromosome or by a shift of template from the F^- chromosome to the *Hfr* fragment during the formation of the unit. If recombination occurs during duplication of an F^- chromosome at least one of the four units produced must be recombinant. We will consider three models by which these units could combine to form the recombinant chromosomes after mating. In model A two recombinant units are produced which pair to form a homozygous recombinant chromosome. In model B only one recombinant unit is produced and emerges as a heterozygous recombinant chromosome. In model C two recombinant units are produced and each of them combines with one of the parental units to form a heterozygous recombinant chromosome.

If certain assumptions are introduced, each model predicts a different segregational pattern. The following assumptions are made:

(*a*) Each nucleus contains one complete chromosome, its duplication resulting in nuclear division.

TABLE 1

NUMBER AND GENETIC PURITY OF RECOMBINANTS AS PREDICTED BY MODEL A, B, AND C IN FIGURE 1

Generation	Number and purity of recombinants		
	A	B	C
0	1-Mixed	1-Mixed	1-Mixed
1	1-Mixed	1-Mixed	1-Mixed
2	1-Pure	1-Mixed	2-Mixed
3	2-Pure	1-Pure	2-Pure
4	4-Pure	2-Pure	4-Pure

(*b*) The increase in the number of F^- cells results from division of tetranucleated cells into binucleated cells. At cell division, the nuclei assort in a specific and regular way, so that two sister nuclei pass into the same daughter cell.

(*c*) The chromosome fragment derived from the *Hfr* parent does not engage in recombination more than once.

(*d*) Recombination does not take place between established chromosomes of an F^- cell.

(*e*) All the descendants of a zygote are viable.

As will be described later, the majority of recombinants in our mating system give segregation results that justify these assumptions.

On the basis of the above assumptions, the three possible models of the segregational patterns of a zygote are diagramatically presented in Figure 1. The number and genetic purity of recombinant cells predicted by the three models are presented in Table 1.

Attention may be called to the following differences between the predictions of the three models. In models A and B a mixed clone does not multiply as such, but gives rise to a pure recombinant clone and nonrecombinant clone. Segregation leading to the production of a pure recombinant clone occurs at the second division after genetic recombination in model A and at the third division in models B and C.

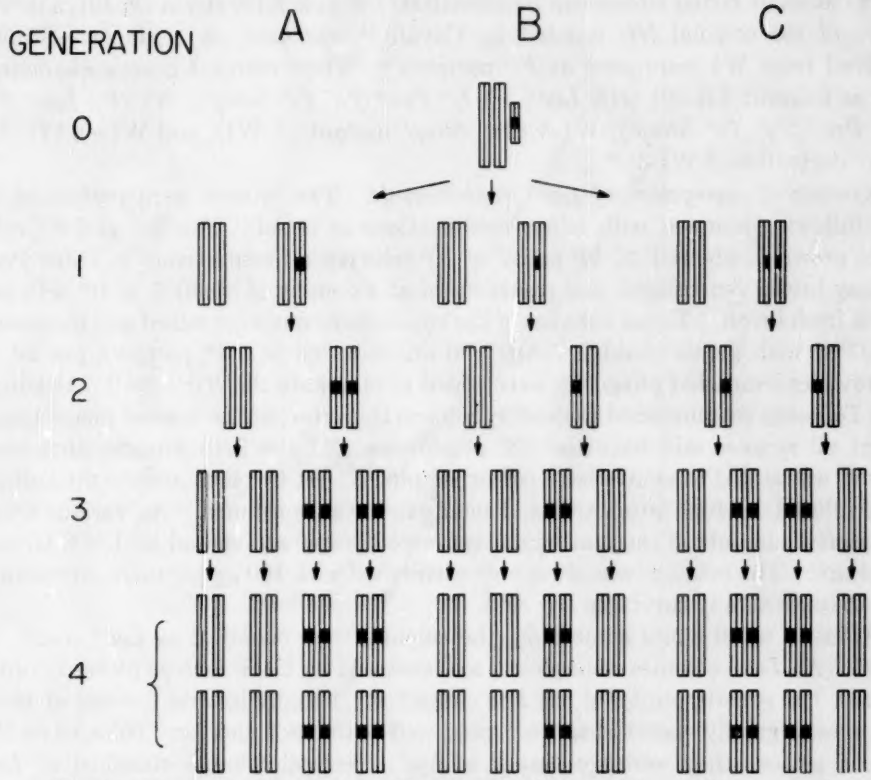

FIG. 1.—Segregational patterns predicted by the three models. A chromosome is represented by two adjacent rectangles, which stand for two units. The chromosomes of an F^- cell are represented by a pair of separate rectangles. A short rectangle represents a chromosome fragment derived from the *Hfr* parent. The *Hfr* fragments are omitted from the figure after the first generation. A filled square is a genetic marker different in the *Hfr* and F^- parents. The descendants of a chromosome that does not participate in recombination are omitted after the second generation.

Model C predicts an increase in the number of mixed clones prior to the division that produces two pure recombinant clones.

These models describe the segregational patterns of single zygotes. The models can be subjected to experimental tests on mass culture if the formation and segregation of zygotes are sufficiently synchronized. The tests consist in plating at intervals the progeny of the zygotes on agar media of such composition that the relevant character of each cell is identified by the character of the corresponding colony.

We shall first describe the properties of our mating system and the kinetics of segregation of recombinants, and then some experimental justification of the assumptions.

Experimental.—The lactose utilization character was selected as the genetic marker to be scored. This character is easily identified by the color of the colonies produced on EMB lactose medium[12]; a pure Lac^+ colony is purple, a pure Lac^- colony is light pink, and a mixed colony Lac^+ and Lac^- is variegated (Lac^v).[13] The system is especially suitable because of the ease with which the cells producing

pure clones or mixed clones can be identified. *E. coli* K12, strain CS 101, a derivative of the original *Hfr* isolated by Cavalli,[14] was used as a donor. Strains derived from W1 were used as F^- partners.[15] Their relevant genetic characters are as follows: CS 101 (*Hfr Lac*$^+$ *T*$^+$ *L*$^+$ *Pro*$^+$ *T5*^{r} *T6*^{s} *Strep*^{s}), W1 (F^- *Lac*$^-$ *T*$^-$ *L*$^-$ *Pro*$^+$ *T5*^{s} *T6*^{s} *Strep*^{s}), W1-3 (*T6*^{r} *Strep*^{r} mutant of W1), and W1−4 (*T*$^+$ *L*$^+$ *Pro*$^-$ derivative of W1-3).[16]

Kinetics of segregation of Lac$^+$ *recombinants:* The crosses were performed in the following manner, with minor modifications as noted. The *Hfr* and F^- cells were grown to about 1×10^8 and 2×10^8 cells per ml respectively in Difco Pennassay broth, centrifuged, and resuspended at a density of about 5×10^8 cells per ml in fresh broth. Equal volumes of the two suspensions were mixed and incubated at 37°C with gentle shaking. After 30 minutes, 2.5×10^{10} particles per ml of ultraviolet-irradiated phage T6 were added to eliminate the *Hfr* cells.[17] Although the *T6*^{s} locus is transferred to most zygotes in this cross, at the time of phage treatment all zygotes still have the *T6*^{r} phenotype. At the 37th minute, antiphage serum was added to neutralize unadsorbed phage. At the 40th minute the culture was diluted 10^5-fold into broth and incubation was continued. At various times thereafter aliquots of the mating culture were diluted and spread on EMB lactose medium. The medium was always supplemented with 100 μg per ml of streptomycin to suppress any surviving *Hfr* cells.

After 18 to 20 hours incubation, the colonies were classified as *Lac*$^+$, *Lac*$^-$, or *Lac*^{v}. All *Lac*$^+$ colonies were picked and streaked on EMB lactose plates in order to test the genetic purity of the *Lac* character. Usually several percent of these colonies originally classified as *Lac*$^+$ produced both *Lac*$^+$ and *Lac*$^-$ colonies on the streak plates; they were reclassified as *Lac*^{v}. Several colonies classified as *Lac*^{v} were also tested by streaking; these invariably produced both *Lac*$^+$ and *Lac*$^-$ colonies.

The results of a cross CS 101 (*Lac*$^+$ *T6*^{s} *Strep*^{s}) and W1-3 (*Lac*$^-$ *T6*^{r} *Strep*^{r}) are presented in Figure 2. The significant features of this experiment may be summarized as follows. The total number of the F^- cells increased from the time of mixing. At first the apparent generation time (doubling time) of the total F^- cells was about 40 minutes, but the cells did not attain exponential growth until about 130 minutes when the generation time had decreased to about 20 minutes. The initial slow growth rate appears to result from the treatment received by the cells before mating and from the initial high concentration of cells in the mating culture, since a similar growth curve was obtained when an unmated F^- culture was treated in the same way.

The number of recombinants that formed *Lac*$^+$ or *Lac*^{v} colonies remained constant from the 40th to the 100th minute. Afterwards it increased at the same rate as the number of F^- cells. Initially most of the recombinants were *Lac*^{v}. At about the 80th minute the number of *Lac*^{v} cells began to decrease, reaching about 50 per cent of the original number at the 100th minute and 10 to 20 per cent at the 130th minute. The decrease of *Lac*^{v} cells was matched by a corresponding increase of *Lac*$^+$ cells until the 100th minute. Zygotes formed by CS 101 and several other strains derived from W1 showed similar kinetics of segregation.

Efficiency of detection of Lac$^+$ *recombinants:* It is especially important for the present experiment to know the efficiency of detection of recombinant clones, that

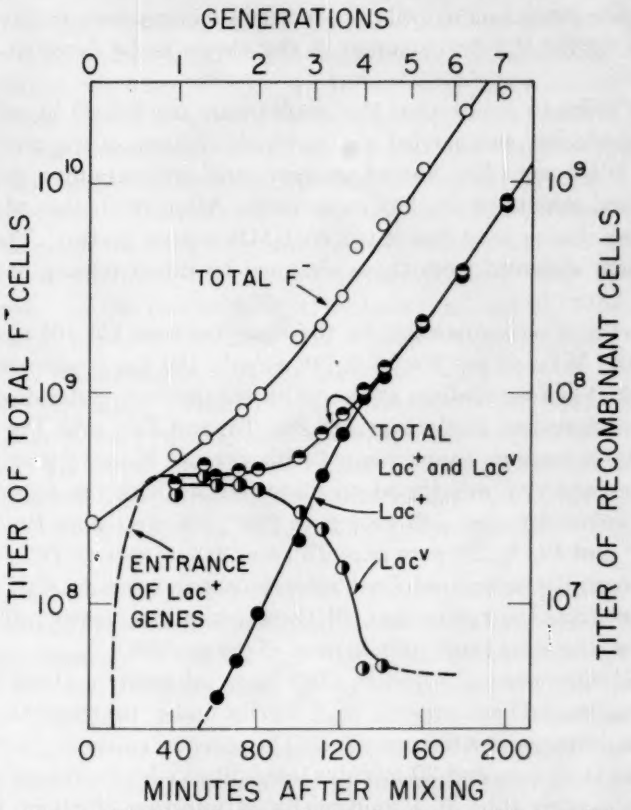

FIG. 2.—Kinetics of segregation of *Lac*$^+$ recombinants. Cultures of CS 101 (*Lac*$^+$ *T6*^{s} *Strep*^{s}) and W1-3 (*Lac*$^-$ *T6*^{r} *Strep*^{r}) were mixed at time zero and incubated. After 30 minutes phage T6 was added to eliminate *Hfr* cells. The mixture was then treated with antiphage serum at the 37th minute to neutralize free phage and diluted at the 40th minute. Aliquots were spread at intervals on EMB lactose medium. The entrance of *Lac*$^+$ genes into *F*$^-$ cells was measured by the blendor technique[11] using a separate culture.

is, clones which contain any *Lac*$^+$ cells. The following experiment indicates that the efficiency of detection is high. The *Lac*$^-$ character of W1 and some of its derivatives is relatively unstable, reverting to *Lac*$^+$ with a frequency of about 1 in 10^7 divisions. Colonies of an unstable strain on EMB lactose medium observed after incubation for 20 hours were typical *Lac*$^-$. On further incubation for two more days, at least five *Lac*$^+$ papillae appeared on every colony. This indicates that *Lac*$^+$ cells arising as a small minority in the colony can be detected efficiently after incubation for three days. By contrast, a strain with a more stable *Lac*$^-$ character, for example W1-4, almost invariably produced colonies without papillae after incubation for three days. Both unstable and stable *F*$^-$ cells were mated with *Hfr* CS 101. After 20 hours incubation, the recombinant cells of both strains produced colonies of similar morphology on EMB lactose medium, suggesting that the metabolic advantage of the *Lac*$^+$ cells over *Lac*$^-$ cells is similar for both strains. On further incubation, however, papillae appeared on all the colonies of the unstable strain but rarely on colonies of the stable strain. This result indicates that

virtually all the recombinant cells produced in consequence of the mating arise early enough during the development of the clones to be detected as variegated colonies.

Finally, in order to assure that the results were not biased by selection on the plates, an experiment was carried out in which aliquots of the mating culture of CS 101 and W1-4 were first spread on agar medium containing glucose, proline, vitamin B_1, and streptomycin, but no lactose. After incubation of the plates for 20 hours, the colonies were replicated on EMB lactose plates. The results were not significantly different from those obtained by direct plating on EMB lactose medium.

Genetic purity of recombinants: In the cross between CS 101 (*Lac*$^+$ *Pro*$^+$ *T5*^{r} *T6*^{s} *Strep*^{s}) and W1-4 (*Lac*$^-$ *Pro*$^-$*T5*^{s} *T6*^{r} *Strep*^{r}), the *Lac*$^+$ colonies formed after plating on EMB lactose medium at the 100th minute were restreaked to determine their purity with respect to the markers *Pro*, *T5*, and *T6*. The *Pro* character was tested on glucose medium supplemented with vitamin B_1 and 0.2 μg per ml of proline. Phage sensitivity was tested on plates seeded with the appropriate phage. Of 250 *Lac*$^+$ colonies tested, 145 were pure *Pro*$^+$, 100 were pure *Pro*$^-$, 5 were mixtures of *Pro*$^+$ and *Pro*$^-$, 220 were pure *T5*^{s}, and 218 were pure *T6*^{s}. The fact that most of the recently segregated *Lac*$^+$ recombinants were pure with respect to the other markers tested suggests that all these genes are incorporated into an F^- chromosome at the same generation in most of the zygotes.

Microscopic observation of F^- cells: To obtain information about the number of nuclei (chromatin bodies) present in F^- cells under the conditions used here, bacteria were examined microscopically. The parental cultures, CS 101 and W1-3, were mixed at time zero and 30 minutes later ultraviolet-irradiated phage T6 was added to the mating tube at a multiplicity of infection of about 1000 to insure prompt lysis of the *Hfr* cells. In this culture the number of *Lac*$^+$ recombinants was 15 per cent of that of input F^- cells. Observations were made on randomly chosen microscopic fields of samples taken at 40, 80, and 130 minutes after mixing. A sample was taken from the F^- culture just before mixing. As a control, aliquots were also taken from a culture in which the *T6*^{s} F^- strain W1, instead of the *Hfr*, was mixed with the F^- strain W1-3. Between 600 to 1000 cells were observed in each sample. The samples were stained by the hydrochloric acid-Giemsa method.[18] Special attention was given to cells with uneven numbers of nuclei.

The results showed that the overwhelming majority of cells contained either two or four nuclei; binucleated cells constitute about two thirds (e.g., 71 per cent at the 80th minute), and tetranucleated ones about one third (e.g., 27 per cent at the 80th minute), of the total cells. The proportion of uninucleated cells was less than two per cent before the 40th minute and less than 0.5 per cent after the 80th minute. Trinucleated cells, which generally showed asymmetric nuclear figures, were less than one per cent of the total cells throughout the experiment. The only difference between the mating suspension and the control culture was that the former contained slightly more cells with more than four nuclei. The proportion of such cells never exceeded one per cent. These observations indicate that the increase in the number of F^- cells results mainly from a division of tetranucleated cells into binucleated cells.

Lederberg[19] and Anderson[20] reported frequent death of the descendants of zy-

gotes. The following results show that in our mating system death of early descendants of zygotes must be infrequent. The same test and control cultures used for the staining experiment were examined. At 100 minutes after mixing the parental cells, aliquots were diluted into broth containing melted one per cent agar at 45°C. The suspension was immediately mounted in a counting chamber, warmed for one hour at 37°C, and examined by phase contrast microscopy to determine the fraction of nonviable cells. The cells which produced microcolonies containing several cells were classified as dividing cells and the cells which remained single or double were classified as nondividing cells. Some nondividing cells may divide after the observation time. In the test culture, 41 (3.1 per cent) out of 1303 cells were nondividing; 21 (1.6 per cent) of these had normal shape, and 20 (1.5 per cent) showed abnormal morphologies. Of the abnormal cells, 12 were abnormal soon after addition to the agar. In the control culture, 13 (2.3 per cent) out of 553 cells were nondividing, 9 (1.6 per cent) having normal shapes and 4 (0.7 per cent) abnormal shapes. Thus the test culture contained only about one per cent more nondividing cells than its control. Since about 10 per cent of the cells in the test culture were derived from zygotes, nondividing cells cannot represent a large fraction of them.

Discussion.—The experimental results can be summarized as follows. (1) *Lac*^{s} cells originating in zygotes do not multiply as such, but segregate into *Lac*$^+$ and *Lac*$^-$ cells. (2) Most of the segregations that produce genetically pure cells takes place between two and 3.5 generations after the entrance of the first *Lac*$^+$ marker into a zygote. (3) Most of the F^- cells are viable and have either two or four nuclei. (4) In most of the zygotes incorporation of *Hfr* markers into an F^- chromosome takes place in a single generation, linked markers being incorporated by a single act.

We shall now discuss the assumptions indicated in the introduction. First of all we assumed that a bacterial chromosome is composed of two units with equivalent genetic information. Although this is nowhere proved, information on DNA as genetic material,[4, 5] on its two-stranded structure,[9] and on its duplication in various organisms[6-8] makes this acceptable as a working hypothesis.

According to assumption (*a*), each nucleus contains one complete chromosome, its duplication resulting in nuclear division. A chromosome is most likely to be located in a nucleus but we are not certain of the ploidy of each nucleus. The present results showing early segregation, however, make it improbable that a nucleus contains two or more copies of *Lac* genes which multiply independently. Experiments on the sensitivity of a bacterium to inactivation by the decay of incorporated radiophosphorus suggest that a nucleus contains a single unit of DNA, and that the survival of the cell requires the integrity of this unit in at least one nucleus.[21] These results suggest that a nucleus is haploid.

Assumption (*b*) states that the increase in the number of F^- cells results from division of tetranucleated cells into binucleated cells and that at cell division the nuclei assort in a specific and regular way, so that two sister nuclei pass into the same daughter cell. The staining experiment supports the assumption as to the number of nuclei. If at cell division the nuclei assort randomly instead of in a regular manner, the number of *Lac*^{s} cells would never decrease. The results also exclude any specific way of assortment that would predict increase or persistence of

Lac^s cells. Thus, sister nuclei must be transmitted to the same daughter cell, as suggested by serial photographic observations on dividing cells.[22] In addition, the fact that the formation of a zygote does not result in the formation of a cell with an uneven number of nuclei suggests that an F^- chromosome doubles whether or not it engages in recombination; that is, a recombinant chromosome does not exist as an "extra" chromosome in the cell.

Assumption (*c*) states that the chromosome fragment derived from the *Hfr* parent does not engage in recombination more than once. If repeated recombination occurred between the *Hfr* fragment or its replicas and the F^- nonrecombinant or heterozygous recombinant chromosome, the cells containing such fragments would be scored as *Lac*^{s} cells as long as they persisted. The fact that *Lac*^{s} cells do not increase in number but segregate to pure *Lac*$^+$ and *Lac*$^-$ cells within a few generations indicates that repeated recombination, if it occurs, must be infrequent.

Assumption (*d*) states that recombination does not take place between the established chromosomes of an F^- cell. Early segregation excludes the possibility of repeated recombination between established recombinant and nonrecombinant chromosomes.

Assumption (*e*) states that all the descendants of a zygote are viable. We have described results showing that nondividing cells cannot represent a majority of cells derived from zygotes. However, it is of critical importance for the present discussion to know whether or not the cells formed immediately after segregation are viable. If a cell which has both *Lac*$^+$ and *Lac*$^-$ chromosomes segregates into one cell with *Lac*$^+$ chromosomes and another with *Lac*$^-$ ones and if the latter fails to produce viable projeny, then that cell would form a pure *Lac*$^+$ colony. The appearance of pure *Lac*$^+$ recombinant colonies might, therefore, result from selective death of *Lac*$^-$ cells. If this were the case, the production of a cell with a pair of recombinant chromosomes would require one generation beyond the formation of an apparently pure *Lac*$^+$ cell, and two generations would be needed for doubling the number of recombinant cells. The rapid increase in *Lac*$^+$ recombinants observed excludes this possibility. Most of the *Lac*$^+$ recombinants must, therefore, have been produced when *Lac*^{s} recombinants segregated into viable *Lac*$^+$ recombinants and nonrecombinants.

Having justified our assumptions, we may now turn to the models between which we wish to discriminate (Fig. 1). Model C, which predicts the multiplication of *Lac*^{s} cells, is incompatible with the experimental results. Models A and B, as far as our experiments are concerned, differ only in their prediction of the time at which pure recombinants appear. Model A predicts that segregation should occur at the second division after genetic recombination, while model B predicts that it should occur at the third division. Our measurements show that most segregations occur between 2 and 3.5 generations after *Lac*$^+$ markers first start to enter the F^- cells. To compare the experimental finding with the predictions, it is necessary to subtract the time required for recombination to occur from the measured interval. How large should this correction be?

First, the distribution of entry times of *Lac*$^+$ markers into mating cells covers half a generation time (Fig. 2), which can be subtracted from the upper limit of the measurement. Second, if a *Lac*$^+$ marker enters an F^- cell at a time when the *Lac* region has already duplicated, recombination might not be possible until the next

generation. If so, an additional generation should be subtracted from the upper limit to the measured segregation time. Finally, the interval between the formation of a recombinant chromosome and the subsequent cell division must itself occupy some part of a generation time. These three considerations lead to the conclusion that the minimal observed segregation time, namely two generations, is an appropriate measure of the interval between recombination and segregation, in good agreement with model A, but not with model B or C. We conclude that recombinant chromosomes are usually already homozygous at the time of formation.

We shall now discuss some results obtained with other mating systems which are pertinent to the present problem. Hayes[17] has described the kinetics of formation of $T^+ L^+$ recombinants in zygotes formed in a cross between *Hfr* H ($T^+ L^+ T1^r T6^s Strep^s$) and P678 ($F^- T^- L^- T1^s T6^r Strep^r$). The number of recombinants stayed constant for the first few generations after mixing the parental cultures and then increased exponentially. The essential features of his result were confirmed in our studies. Under our standard procedure, $T^+ L^+$ recombinants begin to increase at about 100 minutes after mixing, which is also the time at which the number of Lac^+ plus Lac^s cells begins to increase in the system described above. The kinetics of segregation of $T^+ L^+$ recombinants is unknown, but the following result of Hayes[17] is suggestive. In his cross about 75 per cent of $T^+ L^+$ recombinants are $T1^r$. The number of genetically $T1^r$ recombinants begins to increase a few generations after mixing, but phenotypic expression of phage resistance is delayed. The resistance begins to be expressed when the number of the recombinants starts to increase and is fully expressed during the subsequent three or four generations. Hayes interprets his result in the following way. The gene $T1^r$ is recessive to $T1^s$ and, even after segregation, the genetically pure $T1^r$ cells do not express the resistant phenotype immediately. The fact that some cells express the resistant phenotype at the time when the number of recombinants begins to increase indicates that by the time there is an increase in the number of recombinants, some recombinants are genetically pure and have passed the phenotypic lag period. This result again suggests that genetically mixed recombinant cells do not multiply as such.

Lederberg[19] described pedigree analyses of zygotes in a mating system similar to the present one. He found that, usually at the second division, three out of four cells in a clone were pure nonrecombinant type, while the fourth was still generating both parental and recombinant types. These became pure at the third or fourth division. Lederberg also mentions that repeated recombinations were rare events. His results do not generally conflict with the present ones. The results reported by Anderson[20] for a mating system similar to that used by Hayes[17] are quite different, however. Segregation of pure recombinant clones occurred only after three to nine divisions, repeated recombinations were rather frequent, and many cells were nonviable. Also, delayed segregation was described by Lederberg[23] in crosses between *E. coli* strains and by Luria and Burrous[24] in a cross between *E. coli* and *Shigella*. Differences in mating strains, cultural conditions, and technique may underlie these discrepancies. We found slight differences in the kinetics of segregation by different substrains of W1. We also observed considerable differences under different cultural conditions, especially in different media. Differences in techniques may also be im-

portant. In the mass culture method we used, the F^- cells are separated from *Hfr* cells after relatively short contacts, while in pedigree analyses those pairs that remain in contact for a relatively long time tend to be selected. The selection might be nonrandom in other respects if a choice is made on morphological differences. Prolonged contact between mating cells may cause metabolic and divisional disturbances in the zygote.

Results of bacterial cross furnish information about the consequences of recombinational acts but do not tell much about the mechanism. A recombinant chromosome could arise by direct incorporation of an *Hfr* fragment into an F^- chromosome. In this case the present results suggest that both units of the fragment are incorporated simultaneously. Alternatively, recombination may occur by a "copy choice" mechanism[25] during replication. In that case the present results show that the aassortment is different depending on whether or not recombination has occurred. In ordinary replication, each daughter molecule of DNA contains an old and a new unit.[7] A recombinant chromosome, however, according to our results contains only new units.

Levinthal[26] proposed a mechanism of recombination to account for the findings in phage genetics. It is consistent with the basic features of the DNA duplication scheme of Watson and Crick[9] and with our results. According to this mechanism, a recombinant strand of DNA is produced that does not remain associated with either of the parental units from which it is copied, but emerges from a mating in a homozygous, or only locally heterozygous, condition.

Summary.—The kinetics of segregation of several genetic markers in *E. coli* K12 are analyzed. The results suggest that the first recombinant chromosome formed from a zygote is homozygous for most of the genetic markers and that generally only one such recombinant chromosome is formed from each zygote. The implication of this finding for the mechanism of genetic recombination is discussed.

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*EFFECTS OF HEATING DRY BACTERIA AND SPORES ON THEIR PHENOTYPE AND GENOTYPE**

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It has recently been reported that if the cells of *Escherichia coli*, suspended in sucrose solution or dried in air, are heated to 60°C, a high frequency of mutations is produced.[1] The mechanism of the mutagenic action of heat is being studied.[2] The conditions used above for heating offer some protection to the cells; nevertheless viability is lost at temperatures lower than those known to inactivate the deoxyribonucleic acids (DNA).[3] Other methods must be sought if one intends to inflict more frequent genetic damage to DNA without excessive lethality to the cell; and the study of such methods is the object of this paper. It was found that survival at higher temperatures and higher mutation frequencies can be obtained by subjecting vegetative cells (*Escherichia coli*) or spores (*Bacillus subtilis*) to heat in the *dried state in vacuum*. Sufficient survival for determination of mutants was obtained after heating to 135°C to 155°C for 16 min. The corresponding mutant frequencies were 1 to 10 per cent. In the course of this study it was found that in such conditions the vegetative cells of *E. coli* are almost as resistant to heat as the spores of *B. subtilis;* however, the former differ from the latter by exhibiting the phenomenon of pseudoauxotrophy.[1]

Methods.—In series 1C, 24-hr-old (37°C) cultures of *E. coli* (a mutant of strain 15, A.T.C.C. 9723, which is somewhat more resistant to heat) were centrifuged, the sediment transferred to 5-ml glass drying bulbs and dried over P_2O_5 in vacuum. The bulbs were evacuated (2×10^{-2} mm Hg), sealed, totally submerged in boiling water for 3 to 6 hrs (Table 1) and opened. The contents were suspended in 1 ml

TABLE 1

MUTANTS PRODUCED BY HEATING OF DRY CELLS AND SPORES IN VACUUM

Series No.	Material	Heating Conditions	Survival*	Type of Mutants Examined	% Mutants†	Remarks‡
1 C	Dry Cells, *E. coli*	100° 3 hrs	5×10^{-5}	Lactose⁻	3	30 to 90% sectored
		100° 6 hrs	10^{-6}			
2 C	As above	130°, 16 min	7×10^{-5}	....	..	.....
3 C	As above but stored§	130°, 16 min	2×10^{-3}	Auxotrophs	1	Thiamine⁻ (1); Methionine⁻ (2); Histidine⁻ (2); Others (5)
1 S	Dry spores, *B. subtilis*	130°, 16 min	1.4×10^{-6}	....	..	.....
		145°, 16 min	5×10^{-8}			
2 S	As above but stored§	145°, 16 min	2×10^{-5}	Auxotrophs	10	Thiamine⁻ (1); Arginine⁻ (6); Methionine⁻ (6); Histidine⁻ (3); Glutamic acid⁻ (proline⁻) (1); Others (39)
		155°, 16 min	5×10^{-8} to 10^{-7}	Others	11	Pinpoint colonies; Changed morphology; Delayed growth

* Survival of *dried*[4] cells or spores after heating, per cell.
† Per cent of mutants discovered among survivors.
‡ Sectors 1/8 to 1/2 of colony. Numbers of mutants identified are given in parentheses. Auxotrophs other than those specified required casamino acids or yeast extract and were not studied.
§ Stored 90 days after evacuation, before heating.

of nutrient broth and immediately plated on nutrient agar containing 1 per cent lactose and 0.005 per cent triphenyl tetrazolium chloride,[5] for detection of lactose-mutants.

In other experiments, immediately after evacuation (series 2C) or after storage at 4° for 90 days (series 3C) the glass bulbs were totally submerged for 16 min in an oil bath at various temperatures (Table 1), opened, the content suspended in 1 ml Penassay broth (Difco) and immediately plated on Penassay agar, on Penassay agar containing lactose and triphenyl tetrazolium chloride, and on basal agar.[6] The survivors on Penassay agar were tested for the presence of auxtrophs by replica plating on basal agar and Penassay agar.

For heating spores, *Bacillus subtilis* was used because this species can be transformed[6] and therefore the DNA can be investigated to see if it is the carrier of the mutational injury. Strain 23 (wild) of *B. subtilis*, kindly provided by Dr. J. Spizizen, was grown on potato dextrose agar (Difco) for 14 days; the spores were collected and washed. Aliquots containing 10^9 spores were dried and sealed *in vacuum* as described above. The glass bulbs with spores, either immediately after evacuation (series 1S) or after storage at room temp. for 90 days (series 2S) were totally submerged for 16 min in an oil bath at various temperatures (Table 1), opened and tested as described above (with the omission of tetrazolium agar).

To ascertain that the thin pellicle of cells or spores inside the evacuated bulb has indeed reached the temperature of the bath, 1 ml of silicone vacuum pump oil No. 550 (Asco Mfg, Co., Webster, N. Y.) was added to the bulbs after drying but before evacuation. The bulbs were then evacuated, sealed, and heated as before. In these conditions it has been demonstrated by means of an inserted bimetallic thermometer that the pellicle inside the bulb does reach the temperature of the bath within 60 sec. The survival of the dry cells of *E. coli* and of spores of B. *subtilis* after heating was found to be the same as in the absence of oil; a result

which demonstrates that in experiments listed in Table 1 the survival refers to temperatures specified (see below).

Results and Discussion.—Survival: Dried vegetative cells of *E. coli* and spores of *B. subtilis*, when heated in vacuum, exhibit an unusual resistance to high temperatures (Table 1) undoubtedly due to efficient removal of water and O_2 and subsequent slowing down of the heat denaturation reactions. When so dried, the vegetative cells of *E. coli* approach the heat resistance of spores of *B. subtilis*. Storage in vacuum markedly improves heat resistance (Table 1), presumably by improving dehydration or by removing O_2.

Pseudoauxotrophy: The surviving cells undergo phenotypic damage which probably includes heat denaturation of essential enzymes. The damage results in the non-hereditary inability to grow on synthetic medium and delayed growth on full medium; this phenomenon has been termed pseudoauxotrophy.[1]

When the cells of *E. coli* were heated in sucrose solution at 60°C for 2 hrs[1] (survival 10^{-4} on nutrient agar), 99.9 per cent of the survivors were unable to grow on Endo's agar; on nutrient agar only 60 per cent of the colonies that appeared after 26 hours incubation (37°C) were visible at 18 hours, while in an unheated control all colonies appeared after 14 hours. The colonies in heated samples were of various sizes, some as small as pinpoints (non-hereditary). All such heated cells were totally unable to grow on synthetic agar medium (Difco B469) containing glucose, 13 amino acids, 4 nitrogenous bases, and 10 vitamins. On basal agar[6] all unheated controls appeared after 16 hours; in heated samples, however, no colonies appeared at this time, and 89 per cent of the final number appearing on nutrient agar only after 48 hours. If the heating was prolonged to 3 hours, all the survivors (10^{-5} to 2×10^{-6}) were unable to grow on basal agar. The injuries were not hereditary since one transfer on nutrient agar restored the original growth abilities.

Drying the cells in vacuum as described above, by itself did not produce pseudoauxotrophy. However, when the cells dried in vacuum were heated at 130° for 15 min (survival 7×10^{-5} on Penassay agar), suspended in Penassay broth and immediately plated on basal agar, again none of them was able to grow.

It is of interest that the spores of *B. subtilis* (145°, 15 min, survival 4×10^{-7}) did not exhibit pseudoauxotrophy: the survival on basal agar was practically the same as on Penassay agar. This result suggests that there are various protective mechanisms in spores other than just dehydration, since these mechanisms appear to be lacking in dry vegetative cells even though their survival may approach that of spores.

Mutations: As can be seen from Table 1, heating of dry cells or spores resulted in a high proportion of mutants among the survivors. This high proportion makes it unlikely that the mutants were present in the original populations and were merely selected for by heat treatment. However, to further exclude this possibility, the cells of lactose$^-$, histidine$^-$, methionine$^-$, and thiamine$^-$ mutants of *E. coli* were mixed with the cells of the original wild strain in proportions of approx. 50:50, and the survival of each type was determined in the same drying and heating conditions. It was found that mutants lactose$^-$, histidine$^-$, and methionine$^-$ have approximately the same heat resistance as the wild strain, whereas the cells of the mutant thiamine$^-$ are actually less resistant to heat: the proportion, which was initially 44 per cent thiamine$^-$ cells, changed after heating to 9 per cent thiamine$^-$

cells. Since no intermediate selective cultivation between the treatment and the assay of mutant took place, the actual mutant frequency was at least as high as the one observed. Similarly, in the case of *B. subtilis*, the spores of mutants histidine$^-$, methionine$^-$, and thiamine$^-$ were mixed with the spores of the original wild strain in proportions of approx. 50:50, and tested for survival to the same drying and heating conditions. It was found that the spores of the mutants had the same heat resistance as the spores of the wild strain.

Two of the mutants of *B. subtilis* (thiamine$^-$ and methionine$^-$) were used as receptors in transformation experiments.[6] The mutants were transformed to prototrophy by means of DNA from the wild strain 23. This result clearly shows that the carrier of mutational injury is indeed DNA, and that mutation is chromosomal and not of cytoplasmic nature.

After heat treatment certain mutations appear more frequently than others. One example is the mutation to thiamine$^-$ (compare also),[1] both for *E. coli* and *B. subtilis*. It is not unlikely that the structure of the corresponding loci makes them extraordinarily susceptible to injury by heat.[2, 7]

Induction of a high proportion of mutants by heating of dry vegetative cells or spores by itself may be of practical interest. (Compare induction of mutations by nitrous acid recently demonstrated by the author[8] and by others.[8]) More important are the implications as to the mechanism of mutagenesis. The appearance of a sectored lactose$^-$ colony (i.e., part of the colony remains lactose$^+$) implies that the mutation has affected only one strand of a DNA molecule and/or only one nucleus of a multinucleate cell,[9] unless the change has occurred after the first cell division (delayed mutation). In cases where auxotrophs were scored, the survival of the unchanged second strand and/or another nucleus or nuclei (in the same spot on agar) would preclude the detection of auxotroph by the methods employed. Since a high proportion of auxotrophs was detected, this result implies that other strands and/or nuclei became injured so as to be non-reproducible at the time of plating. It is likely that this condition imposes a limit on the proportion of auxotrophs which are theoretically detectable, and that the proportion actually detected is close to such a limit.

The occurrence of mutations without cell division has been reported.[10] However, it cannot be excluded that the mutations occurred during replication of DNA masked by the phenomenon of turnover[11] in the stationary state. In the present experiments it cannot be said whether the actual (final) mutational change resulting from heating was a secondary reaction, i.e., a fault occurring during the first replication of DNA after heating, or whether the final change was already the primary injury[2] induced during heating.[12] Nevertheless, it is certain that here the primary injury by the mutagen (heat) has occurred and has been registered in the conditions in which any replication of DNA was rigorously excluded (dry state, high temperature, spores).

Of further interest is that at least 10 per cent of the mutants in both *E. coli* and *B. subtilis* were very unstable auxotrophs which reverted to prototrophy with a high mutation rate. This finding will be the subject of a separate study.

Summary.—Vegetative cells (*Escherichia coli*) or spores (*Bacillus subtilis*) in the *dried state in vacuum* were heated to 135°C to 155°C for 16 min. In such conditions. the vegetative cells of *E. coli* were almost as resistant to heat as the spores of *B,*

subtilis; however, the former differed from the latter by exhibiting the phenomenon of pseudoauxotrophy. Sufficient survival for determination of mutants was obtained; the mutant frequencies were 1 to 10 per cent (lactose$^-$; auxotrophs). Both stable and unstable mutants were obtained. The mutants of *B. subtilis* could be transformed by DNA from the wild strain. The implications of these findings have been discussed.

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FIELDS OF GEODESICS ISSUING FROM A POINT

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§1. *Introduction.*—We shall be concerned with a 1-parameter family of open Riemannian μ-manifolds H_α of class C^∞, where the parameter α varies on an open interval I. The properties of fields of geodesics issuing from points p_α of the respective manifolds H_α are not fully stated or derived in mathematical literature known to us, at least for the ends we have in mind. This note is designed to fill these needs and will find an application in reference 1.

To simplify the exposition we begin with the more familiar case of a single Riemannian μ-manifold H, $\mu > 1$, leaving to §4 the main theorem of the paper. Let p be an arbitrary fixed point in H. Let $(x^1, \ldots, x^\mu)$ be rectangular coordinates in a Euclidean μ-space E_μ. Set

$$(x^1, \ldots, x^\mu) = \mathbf{x}, \ \|\mathbf{x}\| = (x^i x^i)^{1/2} \qquad (i = 1, \ldots, \mu)$$

employing the summation convention of tensor algebra. We introduce the μ-disk

$$\Delta_\rho^x = \{\mathbf{x} \,|\, \|\mathbf{x}\| < \rho\} \tag{1.0}$$

where ρ is any positive constant.

Let W be an open *coordinate domain* on H which contains p. We suppose that there exists a diffeomorphism

$$\varphi: \Delta_\rho^x \to W: \mathbf{x} \to \varphi(\mathbf{x}) \tag{1.1}$$

of Δ_ρ^x onto W by virtue of which Δ_ρ^x becomes (ref. 2, §0) the *range* of the local coordinates (x). The term "diffeomorphism" in this paper will always mean a diffeomorphism of class C^∞. Since H is supposed to be a Riemannian manifold we include the condition that at the point on W with local coordinates (x)

$$ds^2 = g_{ij}(\mathbf{x}) dx^i dx^j \qquad (i, j = 1, \ldots, \mu), \tag{1.2}$$

where the coefficients g_{ij} are functions of class C^∞ for $\mathbf{x} \in \Delta_\rho^x$, and the invariant quadratic form is positive definite. As far as this paper is concerned H may be supposed identified with W. The compatibility of the above representation φ of W with other representations of open subdomains of W is presupposed in the sense usual for Riemannian manifolds of class C^∞.

We suppose that under the diffeomorphism φ, the point p is represented by the origin in E_μ. We shall also suppose that $g_{ij}(\mathbf{O}) = \delta_{ij}$. If this condition were not satisfied, it would be satisfied for a new set of local coordinates on a neighborhood of p in H, on making a suitable non-singular linear transformation of the initial coordinates (x).

Understanding that $(r^1, \ldots, r^\mu) = \mathbf{r}$ is a contravariant vector associated with the point $(\mathbf{x})$, set

$$f(\mathbf{x}, \mathbf{r}) = [g_{ij}(\mathbf{x}) r^i r^j]^{1/2}. \tag{1.3}$$

A geodesic g on W is represented on Δ_ρ^x by a regular curve

$$t \to \mathbf{x}(t) = (x^1(t), \ldots, x^\mu(t)) \qquad (t \in J) \tag{1.4}$$

of class C^2 (at least), satisfying the Euler equations associated with the integral,

$$\int f(\mathbf{x}, \dot{\mathbf{x}})\, dt.$$

These Euler equations do not uniquely determine the parameterization of g. We shall admit only those representations of geodesics in which the parameter $t = cs$, where s is the arc length measured along the geodesic in the sense of increasing t, and c is a positive constant. We shall limit our study to geodesics issuing from $p \in H$ and shall measure s from p. It is easy to verify the fact that a regular curve, of class C^2, of the form (1.4), represents a geodesic and is of class C^∞, with $t = cs$, if and only if it is a non-null solution of the system of μ differential equations,

$$2 \frac{d}{dt} (g_{hj}(\mathbf{x}) \dot{x}^j) = \frac{\partial g_{ij}}{\partial x^h} (\mathbf{x}) \dot{x}^i \dot{x}^j \qquad (h, i, j = 1, \ldots, \mu). \tag{1.5}$$

On any solution of (1.5)

$$\frac{ds}{dt} = f(\mathbf{x}, \dot{\mathbf{x}}) = c, \tag{1.6}$$

where c is a constant. Moreover $c > 0$ if the solution represents a geodesic, and $c = 0$ if the solution reduces to a point.

§2. *Solutions of (1.5) Vanishing at the Origin.*—Let S be the class of solutions $t \to \mathbf{x}(t)$ of (1.5) such that $\mathbf{x}(t)$ is in $\Delta_\rho^{\mathbf{x}}$ and reduces to the null vector at $t = 0$. In S we include the trivial solution such that $\|\mathbf{x}(t)\| = 0$ for all t. Since the determinant $|g_{hj}(\mathbf{x})| \neq 0$ for $\mathbf{x} \in \Delta\rho$ the following is true. Corresponding to an arbitrary point $(z^1, \ldots, z^\mu) = \mathbf{z}$ in a μ-plane $\mathcal{E}_\mu$ there exists a maximum positive constant $b(\mathbf{z})$, possibly infinite, such that there is a solution of (1.5) in the class S of the form, for fixed (z),

$$t \to \mathbf{A}(t, \mathbf{z}) \qquad (0 \leq t < b(\mathbf{z})) \tag{2.1}$$

with initial derived vector,

$$\mathbf{A}_t(0, \mathbf{z}) = \mathbf{z}. \tag{2.2}$$

If $|\mathbf{z}| \neq 0$ the solution (2.1) of (1.5) represents a geodesic g on H issuing from p with a tangent vector at p given by (z). In this context we regard (z) as a contravariant vector associated with the origin in the coordinate system (x), or equivalently with the point p in H represented by the origin.

The limits $b(\mathbf{z})$ and domain Ω. The limits $b(\mathbf{z})$ are positive, and the function $\mathbf{z} \to b(\mathbf{z})$, defined for every $\mathbf{z} \in \mathcal{E}_\mu$, is lower semicontinuous. This is readily verified. Let Ω be the union of the pairs $(t, \mathbf{z})$ such that

$$0 \leq t < b(\mathbf{z}). \tag{2.3}$$

The set Ω is an open subset of the product of $\mathcal{E}_\mu$ and the positive t-axis. Classical existence theorems on differential equations applied to (1.5) yield the following:

(i) *The function $\mathbf{A}$ is of class C^∞ over Ω.*

For $(t, \mathbf{z}) \in \Omega$ and $\mathbf{z}$ fixed, set $\mathbf{x}(t) = \mathbf{A}(t, \mathbf{z})$. With $k \geq 0$ set $\mathbf{x}(kt) = y(t)$. Then $t \to \mathbf{y}(t)$ is a solution of (1.5) for t in an appropriate maximal open subinterval J of the positive t-axis. The initial derived vector on this solution is $k\mathbf{z}$. Such a solution of (1.5) is given by the function $t \to \mathbf{A}(t, k\mathbf{z})$ for the given fixed k and $\mathbf{z}$, and for $t \in J$. Hence we have the fundamental relation

$$\mathbf{A}(t, k\mathbf{z}) = \mathbf{A}(kt, \mathbf{z}) \qquad (t, k\mathbf{z}) \in \Omega. \tag{2.4}$$

This relation leads to the following:

(ii) *For $k \geq 0$, the pair $(t, k\mathbf{z})$ is in Ω if and only if $(kt, \mathbf{z})$ is in Ω. If $(t, \mathbf{z})$ is in Ω then for $(0 \leq k \leq 1)$, $(kt, \mathbf{z})$ and $(t, k\mathbf{z})$ are in Ω.*

Let $\|\mathbf{z}\|$ be the Euclidean length of the vector $\mathbf{z}$ in the μ-plane $\mathcal{E}_\mu$. Since $g_{ij}(\mathbf{O}) = \delta_{ij}$ it follows that $f(\mathbf{O}, \mathbf{z}) = \|\mathbf{z}\|$ when $\|\mathbf{z}\| \neq 0$. When $\|\mathbf{z}\| = 0$, $f(\mathbf{O}, \mathbf{z})$ is not defined. The set of points $\mathbf{z} \in \mathcal{E}_\mu$ such that $\|\mathbf{z}\| = 1$, is compact. Since the function $\mathbf{z} \to b(\mathbf{z})$ is lower-semicotninuous one can affirm the existence of the

$$min\ (b(\mathbf{z}) \mid \|\mathbf{z}\| = 1) = \eta, \qquad \text{(introducing } \eta) \tag{2.6}$$

The minimum η is positive. We shall prove (iii).

(iii) *The pairs* $(t, \mathbf{z})$ *of the form* $(1, \mathbf{z})$ *for which* $\|\mathbf{z}\| < \eta$ *are in* Ω.

Pairs $(1, \mathbf{z})$ for which $\|\mathbf{z}\| = 0$ are clearly in Ω. Suppose then that $0 < \|\mathbf{z}\| < \eta$. Note that

$$(1, \mathbf{z}) = \left(\frac{\|\mathbf{z}\|}{\|\mathbf{z}\|}, \mathbf{z}\right) \tag{2.7}$$

By (*ii*) the right-hand pair in (2.7) is in Ω if the pair

$$\left(\|\mathbf{z}\|, \frac{\mathbf{z}}{\|\mathbf{z}\|}\right) \tag{2.8}$$

is in Ω. But the norm of the vector in this pair is 1, and the numerical value of the first element is less than η. By definition of η the pair (2.8) is in Ω, and (iii) follows.

We shall presently use the following fact. If $\|\mathbf{a}\| \neq 0$ and if one sets $\mathbf{x}(t) = \mathbf{A}(t, \mathbf{a})$, then for $0 \leq t < b(\mathbf{a})$,

$$\frac{ds}{dt} = f(\mathbf{x}(t), \dot{\mathbf{x}}(t)) = f(\mathbf{x}(0), \dot{\mathbf{x}}(0)) = f(\mathbf{O}, \mathbf{a}) = \|\mathbf{a}\|. \tag{2.9}$$

§3. *A Geodesic Isometry.*—Let a be a positive constant and let Δ_a^z be the subset of points $\mathbf{z}$ in $\mathcal{E}_\mu$ such that $\|\mathbf{z}\| < a$. With η defined as in (2.6) we shall map Δ_η^z into the μ-plane E_μ of coordinates $\mathbf{x}$ by setting

$$\mathbf{A}(1, \mathbf{z}) = \mathbf{x} \qquad (\mathbf{z} \in \Delta_\eta^z) \tag{3.1}$$

or, in terms of the components A^i of $\mathbf{A}$, by setting

$$A^i(1, \mathbf{z}) = x^i \qquad (i = 1, \ldots, \mu) \tag{3.2}$$

The mapping (3.2) is well-defined for $\mathbf{z} \in \Delta_\eta^z$ in accord with (iii) of §2. We shall show that

$$\frac{\partial A^i}{\partial z^j}(1, \mathbf{z}) \,|\, (\|\mathbf{z}\| = 0) = \delta_j^i \qquad (i, j = 1, \ldots, \mu) \tag{3.3}$$

Proof of (3.3): If $0 \leq t \leq 1$ and $\|\mathbf{z}\| < \eta$ the pair $(1, t\mathbf{z})$ is in Ω according to (ii) and (iii), and

$$A^i(1, t\mathbf{z}) = A^i(t, \mathbf{z}) \qquad (i = 1, \ldots, \mu). \tag{3.4}$$

by virtue of (2.4). Differentiating the members of (3.4) with respect to z^j gives

$$\frac{\partial A^i}{\partial z^j}(1, t\mathbf{z}) = \frac{\partial A^i}{\partial z^j}(t, \mathbf{z}) \qquad (i, j = 1, \ldots, \mu). \tag{3.5}$$

Differentiating the members of (3.5) with respect to t and setting $t = 0$ gives

$$\frac{\partial A^i}{\partial z^j}(1, \mathbf{z}) \,|\, (\|\mathbf{z}\| = 0) = \frac{\partial^2 A^i}{\partial t \partial z^j}(t, \mathbf{z}) \,|\, (t = 0)$$

$$\text{(by (2.2))}. \qquad = \frac{\partial^2 A^i}{\partial z^j \partial t}(t, \mathbf{z}) \,|\, (t = 0) = \frac{\partial z^i}{\partial z^j} = \delta_j^i. \tag{3.6}$$

This establishes (3.3).

Let X denote the coordinate range Δ_ρ^x in E_μ. To simplify statement (i) which follows we regard X as a Riemannian manifold X^* with a differential form given by (1.2).

(i) *If ϵ is a sufficiently small positive constant the mapping*

$$\mathbf{z} \to \mathbf{A}(1, \mathbf{z}) \qquad (\mathbf{z} \in \Delta_\epsilon^x) \tag{3.7}$$

is a diffeomorphism of Δ_ϵ^x into X^* in which a ray λ issuing from the origin in Δ_ϵ^x is mapped isometrically onto a geodesic arc in X^* issuing from the origin.

Let $\epsilon < \eta$ be chosen as a positive number so small that the mapping (3.7) is a diffeomorphism. This is possible because of (3.3).

Let $\mathbf{a}$ now be a point in $\mathcal{E}_\mu$ such that $\|\mathbf{a}\| = 1$. The ray λ may be given as a set of points

$$\{\mathbf{z} \,|\, \mathbf{z} = t\mathbf{a},\ 0 \leq t < \epsilon\}.$$

The image of $\mathbf{z} = t\mathbf{a}$ under the diffeomorphism (3.7) is the point

$$\mathbf{x} = \mathbf{A}(1, t\mathbf{a}) = A(t, \mathbf{a}) \qquad (0 \leqq t < \epsilon)$$

This is a point on the geodesic arc γ in X^* with initial derived vector $\mathbf{a}$, and distance s, measured along γ from the origin, such that $s = t\|\mathbf{a}\| = \|z\|$. Cf. 2.9. This establishes (i)

Since the diffeomorphism φ in (1.1) may be regarded as an isometry of X^* onto H, the preceding statement (i) gives us the following. Cf. ref. 3, pp. 97–100.

LEMMA 3.1. *Corresponding to the point $p \in H$ and a sufficiently small positive constant ϵ, there exists a diffeomorphism*

$$\psi : \Delta_\epsilon^z \to H;\ \mathbf{z} \to \psi(\mathbf{z})$$

such that each ray λ in Δ_ϵ^z issuing from the origin with $0 = \|\mathbf{z}\| < \epsilon$ on λ is mapped isometrically onto a geodesic arc issuing from $p \in H$.

This lemma is preliminary to the principal theorem of §4. We *term the mapping ψ in the lemma a radial-geodesic isometry of Δ_ϵ^z onto a geodesic μ-disk in H with pole p.*

§4. A 1-Parameter Family of μ-Manifolds H_α.—We shall consider a 1-parameter family of μ-manifolds regularly embedded in a Riemannian n-manifold M of class C^∞ with $n = \mu + 1$. We suppose that the parameter α varies on an open interval I which includes $\alpha = 0$. Our family of μ-manifolds H_α is defined by a diffeomorphism

$$\Phi : \Delta_\rho^x \times I \to M;\ \mathbf{x} \times \alpha \to p(\mathbf{x}, \alpha), \tag{4.0}$$

where $p(\mathbf{x}, \alpha)$ is a point in M, and where, for fixed $\alpha \in I$, Φ reduces to a diffeomorphism,

$$\Phi_\alpha : \Delta_\rho^x \to M;\ \mathbf{x} \to p(\mathbf{x}, \alpha) \tag{4.1}$$

which serves to define H_α as the image of Δ_ρ^x under Φ_α. Since our principal lemma is local in character we can suppose without loss of generality that Φ is a map *onto* M. Thus M is given as a single coordinate domain. The sets $(x^1, \ldots, x^\mu, \alpha)$ in $\Delta_\rho^x \times I$ are thus local coordinates on M.

Let a Riemannian metric be assigned the manifolds H_α by restriction of the first fundamental form on M. Thus at the point $p(\mathbf{x}, \alpha) \in H_\alpha$

$$ds^2 = g_{ij}(\mathbf{x}, \alpha)dx^i dx^j \qquad (i, j = 1, \ldots, \mu). \tag{4.2}$$

where the coefficients g_{ij} are functions of class C^∞ on $\Delta_\rho^x \times I$, and for each $\alpha \in I$ the form (4.2) is positive definite.

A curve κ traversing the family $\{H_\alpha | \alpha \in I\} = \Lambda$. Let κ be a simple, regular arc of class C^∞ on M, meeting each manifold H_α in a single point p_α and not tangent to H_α at p_α. With κ so defined the principal theorem of this paper follows.

THEOREM 4.1. *Let κ be a regular curve of class C^∞ traversing Λ. Corresponding to a sufficiently small positive constant ϵ and a sufficiently small subinterval J of I containing $\alpha = 0$, there exists a diffeomorphism Ψ of $\Delta_\epsilon^z \times J$ into M which for fixed $\alpha \in J$ reduces to a radial-geodesic isometry Ψ_α of Δ_ϵ^z onto a geodesic μ-disk in H_α with pole $H_\alpha \cap \kappa$.*

Since the sets $(x^1, \ldots, x^\mu, \alpha)$ in $\Delta_\epsilon^z \times I$ are admissible coordinates on M and the arc κ is not tangent to any of the manifolds H_α, on which α is constant, κ has a representation $x^i = \varphi^i(\alpha) : i = 1, \ldots, \mu$, of class C^∞ for $\alpha \in I$. Without loss of generality we can suppose that each of the functions φ^i vanishes. Were that not the case a change of local coordinates of the form

$$y^i = x^i - \varphi^i(\alpha) \qquad (i = 1, \ldots, \mu)$$

would bring it about. Such a change would call for a proper new choice of Δ_ρ^y and interval I. We shall suppose however that κ is represented by the interval I of the α-axis in the space $E_\mu \times I$.

The proof of the theorem is similar to the proof of Lemma 3.1. It requires supplementary consideration of how the mappings introduced in §§1–3 depend upon α.

Without loss of generality in the proof of the theorem we can suppose that

$$g_{ij}(\mathbf{O}, \alpha) = \delta_{ij} \qquad (i, j = 1, \ldots, \mu) \tag{4.3}$$

provided α is restricted to some sufficiently small neighborhood of $\alpha = 0$. In fact if (4.3) did not hold for some such neighborhood of $\alpha = 0$, then for some sufficiently small constant $\delta > 0$ it would be possible, for each $\alpha \in (-\delta, \delta)$, to make a non-singular, linear transformation of coordinates

$$L_\alpha : y^i = b_{ij}(\alpha)x^j \tag{4.4}$$

such that the coefficients b_{ij} are functions of class C^∞ for $\alpha \in (-\delta, \delta)$ and the condition (4.3) holds for the transformed coefficients. The transformations (4.4) are readily defined, making use of a modified Lagrangian method of reduction of a quadratic form. The reader is referred to a proof of Lemma 10.1 in Ref. 4 for an application of such a method.

We assume then that (4.3) holds for $\alpha \in I$.

The differential equations which here replace (1.5), have the same form, except that $g_{ij}(\mathbf{x}, \alpha)$ replaces $g_{ij}(\mathbf{x})$. Limits $b(\mathbf{z}, \alpha)$ are here defined for each $\alpha \in I$ and $\mathbf{z} \in \mathcal{E}_\mu$, as was $b(\mathbf{z})$ in §2. The resulting function $(\mathbf{z}, \alpha) \to b(\mathbf{z}, \alpha)$ is lower semi-continuous on the product $\mathcal{E}_\mu \times I$.

Let Ω^* be the union of the triples $(t, \mathbf{z}, \alpha)$ for $0 \leq t < b(\mathbf{z}, \alpha)$ and $\alpha \in I$. The function $\mathbf{A}$ with values $\mathbf{A}(t, \mathbf{z}, \alpha)$ and domain of definition Ω^*, is defined for fixed $\alpha \in I$, as was the function $\mathbf{A}$ in §2. The new function is of class C^∞ over Ω^*, and the identity

$$\mathbf{A}(t, k\mathbf{z}, \alpha) = \mathbf{A}(kt, \mathbf{z}, \alpha) \tag{4.5}$$

is valid for $(t, k\mathbf{z}, \alpha) \in \Omega^*$ and $k \geq 0$. As previously $(t, k\mathbf{z}, \alpha)$ is in Ω^* if and only if $(kt, \mathbf{z}, \alpha)$ is in Ω^*.

Let K be a relatively compact subinterval of I that contains $\alpha = 0$. Set

$$\min\,(b(\mathbf{z}, \alpha)\,|\,\|\mathbf{z}\| = 1,\ \alpha \in \bar{K}) = \eta. \tag{4.6}$$

Then $\eta > 0$ and (iii) of §2 takes the following form:

The triples $(t, \mathbf{z}, \alpha)$ *of the form* $(1, \mathbf{z}, \alpha)$ *for which* $\|\mathbf{z}\| < \eta$ *and* $\alpha \in K$ *are in* Ω^*.

For each $\alpha \in K$ we map Δ_η^z into E_μ by setting

$$\mathbf{A}(1, \mathbf{z}, \alpha) = \mathbf{x}, \tag{4.7}$$

and show as in §3. that the Jacobian of the functions $A^1, \ldots, A^\mu$ with respect to the variables $(z^1, \ldots, z^\mu)$ equals 1 when $(t, \mathbf{z}, \alpha) = (1, \mathbf{O}, 0)$. Reasoning as in the proof of Lemma 3.1 we conclude as follows:

For a sufficiently small positive ϵ and choice of J as subinterval of K, the mapping

$$\Delta_\epsilon^z \times J \to \Delta_\rho^x \times J\colon (\mathbf{z}, \alpha) \to (\mathbf{A}(1, \mathbf{z}, \alpha), \alpha)$$

followed by the mapping Φ of (4.0) gives Ψ of Theorem 4.1.

[1] Morse, Marston, "The existence of polar non-degenerate functions on differentiable manifolds," *Annals of Math.*, **71** (1960).

[2] Morse, Marston, "Differentiable mappings in the Schoenflies Theorem," *Compositio Mathematica*, **14**, (1959).

[3] Seifert, H., and W. Threlfall, *Variationsrechung im Grossen*, ed. B. G. Teubner (Leipzig and Berlin: Teubner, 1938), pp. 97–100.

[4] Morse, Marston, "Functional topology and abstract variational theory," in *Memorial des Sciences Mathematiques* (Paris: Gauthier-Villars, 1938), p. 44.

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*FUNDAMENTAL GROUPS ON A LORENTZ MANIFOLD**

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1. Introduction.—Let $\mathcal{U}$ be a topological space and x a point in $\mathcal{U}$. The absolute homotopy group $\pi_n(\mathcal{U}, x)$ in dimension n may be derived from the set $M_n(\mathcal{U}, x)$ of maps $f\colon I^n, \dot{I}^n \to \mathcal{U}, x$, where I^n denotes the unit n-cube and $\dot{I}^n$ its boundary. More precisely, $\pi_n(\mathcal{U}, x)$ can be regarded as the set of equivalence classes in $M_n(\mathcal{U}, x)$ under homotopy, with multiplication induced by multiplication in $M_n(\mathcal{U}, x)$.[1] Now let us suppose that the space $\mathcal{U}$ carries some additional structure permitting us to distinguish a proper subset $X_n(\mathcal{U}, x)$ of $M_n(\mathcal{U}, x)$ by its intrinsic properties. Two elements f, g of $M_n(\mathcal{U}, x)$ may be called *homotopic in* $X_n(\mathcal{U}, x)$ if there exists a homotopy $F\colon I^n \times I, \dot{I}^n \times I \to \mathcal{U}, x$ between f and g such that, for every t in I, F restricted to $I^n \times t, \dot{I}^n \times t$ gives an element of $X_n(\mathcal{U}, x)$. This defines an equivalence relation on $X_n(\mathcal{U}, x)$ and determines a set $\xi_n(\mathcal{U}, x)$ of equivalence classes. If $X_n(\mathcal{U}, x)$ is closed under multiplication in $M_n(\mathcal{U}, x)$, then this induces a multiplication on $\xi_n(\mathcal{U}, x)$. The cases of interest are those where

$\xi_n(\mathfrak{V}, x)$ is a group, and where the natural homomorphism i^*: $\xi_n(\mathfrak{V}, x) \to \pi_n(\mathfrak{V}, x)$ induced by the inclusion map i: $X_n(\mathfrak{V}, x) \to M_n(\mathfrak{V}, x)$ fails to be an isomorphism. The new groups are not topological invariants of $\mathfrak{V}$, but depend on the additional structure of the space which has been used to distinguish $X_n(\mathfrak{V}, x)$. It is precisely for this reason that they can be significant in the classification and study of the given spaces.

We are concerned with the case where $\mathfrak{V}$ is a Lorentz manifold, i.e., a differentiable manifold of some class C^p, $2 \leq p \leq \infty$, carrying a second order symmetric covariant tensor field of signature $(2 - n)$ and class C^2. We suppose in fact that the given quadratic form has 1 positive and $(n - 1)$ negative eigenvalues everywhere on $\mathfrak{V}$, n being the dimension of $\mathfrak{V}$. This gives an indefinite Riemannian metric on $\mathfrak{V}$ and enables us to distinguish *time-like*, *null*, and *space-like* tangent vectors, according as their length is positive, zero, or imaginary, respectively. This circumstance makes it possible to define suitable subsets $X_n(\mathfrak{V}, x)$ leading to new homotopy groups.

2. New Fundamental Groups.—In the present note we restrict ourselves to homotopy in dimension one. An arbitrary map f: $I \to \mathfrak{V}$ will be referred to as a *path*, *starting* at $f(0)$ and *ending* at $f(1)$. If f is piecewise differentiable, it determines a unique tangent vector at $f(t)$ for every regular t in I, and two distinct tangent vectors for every singular t in I. The path is called *time-like* if all these tangent vectors are time-like. The set of time-like vectors in every tangent vector space to $\mathfrak{V}$ consists of two disconnected components. Two time-like vectors belonging to the same tangent vector space will be called *equally* or *oppositely directed*, depending on whether they belong to the same connected component or not. If f is a time-like path, a singular point t in I such that the two corresponding tangent vectors at $f(t)$ are oppositely directed will be called a *corner*. We now define a *q-loop based at x* (q a non-negative integer) to be a time-like path f satisfying the following conditions: (*i*) $f(t) = x$ if and only if t belongs to the set $\{0, 1\}$, (*ii*) the two tangent vectors to f at x are equally directed, (*iii*) f has exactly q corners. If f is an arbitrary path starting at x, the path ff^{-1} (which starts and ends at x) will be referred to as a *sting based at x*. Let $T_q(\mathfrak{V}, x)$ denote the subset of $M_1(\mathfrak{V}, x)$ generated by the totality of q-loops and stings, based at x. In other words, the elements of $T_q(\mathfrak{V}, x)$ are finite products involving q-loops and stings, based at x. This gives us an infinite collection of distinguished subsets of $M_1(\mathfrak{V}, x)$, and one verifies immediately that these lead to groups by the process described in Section 1. We will denote these groups by $\tau_q(\mathfrak{V}, x)$.

3. Natural Homomorphisms.—The inclusion maps i_q: $T_q(\mathfrak{V}, x) \to M_1(\mathfrak{V}, x)$ induce homomorphisms i_q^* of the corresponding groups. Apart from this one can show[2]

THEOREM 1. *For every $q \geq 0$ there exists a natural homomorphism h_q making the following diagram commutative:*

$$\begin{array}{ccc} \tau_q(\mathfrak{V}, x) & \xrightarrow{h_q} & \tau_{q+2}(\mathfrak{V}, x) \\ & {\scriptstyle i_q^*} \searrow \quad \swarrow {\scriptstyle i_{q+2}^*} & \\ & \pi_1(\mathfrak{V}, x) & \end{array}$$

It is of interest to note that the maps shown in this diagram are neither one-to-

one nor onto, in general, as can be seen by considering various examples. One observes further that the new groups give rise to two direct sequences, viz.[3]

$$\tau_0 \xrightarrow{h_0} \tau_2 \rightarrow \ldots \quad \& \quad \tau_1 \xrightarrow{h_1} \tau_3 \rightarrow \ldots$$

Let us designate the corresponding direct limits by $\tau+$ and $\tau-$, respectively. The maps $i_q{}^*$ determine now two homomorphisms $i_\pm$: $\tau_\pm \rightarrow \pi_1$, and one can prove

THEOREM 2. *$i+(i_-)$ maps $\tau+(\tau_-)$ isomorphically onto the normal subgroup $\pi_1{}^+(\pi_1{}^-)$ of π_1, generated by loops whose holonomy transformation preserves (interchanges) the two components of time-like tangent vectors. If $\mathfrak{V}$ is Lorentz orientable,[4] $\pi_1{}^+ = \pi_1$ and the groups $\pi_1{}^-$, $\tau_q(q$ odd) vanish. Otherwise $\pi_1{}^+$ is a subgroup of index 2 and $\pi_1{}^- = \pi_1$.*

4. Components and Global Equivalence.—So far we have considered the new groups at a fixed base point x. While $\pi_1(\mathfrak{V}, x)$ does not depend on x as an abstract group, $\tau_q(\mathfrak{V}, x)$ does in general depend on x. We are consequently led to define a *component* of $\mathfrak{V}$ to be a maximal connected subset $\mathfrak{U}$ of $\mathfrak{V}$ such that x, y in $\mathfrak{U}$ implies the existence of isomorphisms ϕ_q: $\tau_q(\mathfrak{V}, x) \rightarrow \tau_q(\mathfrak{V}, y)$, $q \geq 0$, which commute with the maps h_q. The given algebraic structure defines thus a partition of $\mathfrak{V}$ into a number of disjoint subsets. If x is a point of $\mathfrak{V}$, we will denote by $\{x\}$ the component to which it belongs. It is not easy to give a geometric characterization of $\{x\}$, except when $\tau_0(\mathfrak{V}, x)$ is nontrivial. In this case we have

THEOREM 3. *If $\tau_0(\mathfrak{V}, x)$ is nontrivial, then $\{x\}$ is the totality of points belonging to the image of O-loops based at x. Moreover, if z belongs to the boundary of $\{x\}$, then $\tau_0(\mathfrak{V}, z)$ is trivial.*

Some additional information about these components is given by

THEOREM 4. *If $\tau_0(\mathfrak{V}, x)$ is nontrivial, there exists a natural epimorphism k: $\tau_0(\mathfrak{V}, x) \rightarrow \pi_1(\{x\}, x)$.*

The new groups also give rise to an equivalence relation for Lorentz manifolds. Thus $\mathfrak{V}$ and $\mathfrak{V}^*$ may be called *equivalent* if there exists a differentiable homeomorphism f: $\mathfrak{V} \rightarrow \mathfrak{V}^*$ such that, for every x in $\mathfrak{V}$, there exist isomorphisms ϕ_q: $\tau_q(\mathfrak{V}, x) \rightarrow \tau_q(\mathfrak{V}^*, f(x))$, $q \geq 0$, which commute with the maps h_q. We observe that this notion of equivalence is *global* in the sense that it renders all Lorentz manifolds of the same dimension locally equivalent, and it is also *nontrivial on n-cells* ($n > 2$), in the sense that there exist numerous nonequivalent Lorentz structures on R^n.

The concepts introduced in this note bring to light a new type of global property—measured by the τ-groups—which has no analogue in Riemannian geometry with definite metric. One is naturally interested to know more about these global properties, and their dependence on curvature.

In conclusion we will state

THEORUM 5. *If $\mathfrak{V}$ is flat and complete,[5] then the maps h_0 are monomorphisms and the maps h_q $q > 0$, are isomorphisms.* This shows, in particular. that the kernels of h_q measure global effects of curvature.

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[1] Cf., Hilton, P. J., *Introduction to Homotopy Theory* (Cambridge University Press, 1953), p. 5.

[2] Proofs of theorems mentioned in this note will be published elsewhere, with examples.

[3] We write τ_q for $\tau_q(\mathfrak{U}, x)$, etc., where the meaning is sufficiently clear.

[4] $\mathfrak{U}$ is said to be *Lorentz orientable* if the bundle of time-like tangent vectors is disconnected.

[5] $\mathfrak{U}$ is *complete* if every geodesic is extendible to infinite values of the canonical parameter for an arbitrary canonical parametrization. The latter is one which causes the tangent vectors at distinct points to be parallel with respect to the geodesic.

A SIMPLE PROOF OF THE CUP PRODUCT REDUCTION THEOREM

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The purpose of this note is to give a simple proof of the cup product reduction theorem[1] and, at the same time, to clarify the relation between group extensions and cohomology classes. The theorem is most easily stated and proved using Yoneda's definition[2] of *Ext*. This will be done in §3. In §4, I will show that the theorem proved in §3 is really the classical cup product reduction theorem.

1. Preliminaries. For any group π, $Z(\pi)$ will denote its integral group ring. Let $\mathfrak{C}$ be the relative category[3] whose objects and maps are $Z(\pi)$-modules and maps but in which a sequence is only considered exact if it splits over Z.[4] Throughout this note, "$Ext_{\mathfrak{C}}$" will denote the functor *Ext* for the category $\mathfrak{C}$. The elements of $Ext^n_{\mathfrak{C}}$ (A, C) are equivalence classes of Z-split sequences

$$E: \quad 0 \to C \to E_{n-1} \to \ldots \to E_0 \to A \to 0.$$

I will denote the equivalence class of E by $[E]$. I will refer to the natural pairing[5]

$$Ext^i_{\mathfrak{C}}(A, B) \otimes Ext^j_{\mathfrak{C}}(B, C) \to Ext^{i+j}_{\mathfrak{C}} \quad (A, C)$$

as the Yoneda product and denote it by simple juxtaposition ($\alpha \otimes \beta \to \alpha\beta$).

Recall that the notion of a relatively projective module in the category $\mathfrak{C}$ is exactly the notion of a weakly projective module over $Z(\pi)$.[6]

LEMMA 1. *In the sequence*

$$E: \quad 0 \to C \to E_{n-1} \to \ldots \to E_0 \to A \to 0,$$

assume that the modules E_i are all (weakly) projective. Then the map $Ext^i_{\mathfrak{C}}(C, D) \to Ext^{i+n}_{\mathfrak{C}}(A, D)$ by $\alpha \to [E]\alpha$ is an isomorphism for $i > 0$.

Proof: The sequence E is the Yoneda product of short exact sequences $0 \to C \to E_{n-1} \to X_{n-2} \to 0$, $0 \to X_{n-2} \to E_{n-2} \to X_{n-3} \to 0$, ... Consequently, it is sufficient to prove the lemma for the case $n = 1$. Since E_0 is weakly projective, $Ext^j_{\mathfrak{C}}(E_0, D) = 0$ for $j > 0$. Therefore, the exact sequence[7] for *Ext* shows that the connecting homomorphism $Ext^i_{\mathfrak{C}}(C, D) \to Ext^{i+1}_{\mathfrak{C}}(A, D)$ is an isomorphism for $i > 0$. However, this connecting homomorphism is defined to be the map $\alpha \to [E]\alpha$.

2. Group extensions. Suppose $1 \to H \to G \to \pi \to 1$ is an extension of groups. I will show how to associate with this extension an element u of $Ext^2_{\mathfrak{C}}(Z, A)$ where $A = H/[H, H]$ is the factor commutator group of H. The group π acts on A in the

usual way. That is, if $y \in G$ represents $x \in \pi$, and $b \in H$ represents $a \in A$, then $yby^{-1} \in H$ represents $xa \in A$. Choose a projective resolution.

$$P: \quad \ldots \to P_2 \to P_1 \to P_0 \to Z \to 0$$

for Z over $Z(G)$. Then P is also a projective resolution for Z over $Z(H)$. The collapsed sequence

$$\ldots \to P_2/H \to P_1/H \to P_0/H \to Z \to 0$$

is a chain complex over $Z(\pi)$. Its homology groups are the groups $H_i(H, Z)$ for $i > 0$. Now, there is a natural isomorphism[8]

$$H_1(H, Z) \approx A. \tag{1}$$

Consequently, if X_1 is the cokernel of the map $P_2/H \to P_1/H$, we get an exact sequence

$$0 \to A \to X_1 \to P_0/H \to Z \to 0 \tag{2}$$

If we choose another projective resolution P′ for Z over $Z(G)$, there is a map[9] $P' \to P$. This induces a map of the sequences (2) which is the identity on A and Z. Thus the resulting sequences (2) are equivalent and so define the same element $u \in Ext^2_{\mathfrak{C}}(Z, A)$. I will refer to u as the characteristic class of the group extension.

More generally, suppose we have a map of group extensions

$$\begin{array}{ccccccccc} 1 & \to & H' & \to & G' & \to & \pi & \to & 1 \\ & & \downarrow h & & \downarrow g & & \downarrow = & & \\ 1 & \to & H & \to & G & \to & \pi & \to & 1 \end{array}$$

Let P' be a projective resolution for Z over $Z(G')$. The group G' acts on P through the map g. Thus there is a map[9] $P' \to P$. This induces a map

$$\begin{array}{ccccccccc} 0 & \to & A' & \to & X_1' & \to & P_0'/H' & \to & Z \to 0 \\ & & \downarrow h' & & \downarrow & & \downarrow & & \downarrow = \\ 0 & \to & A & \to & X_1 & \to & P_0/H & \to & Z \to 0 \end{array}$$

Consequently, if u', u are the characteristic classes of the two extensions, we have $h^*(u') = u$ where h^*: $Ext^2_{\mathfrak{C}}(Z, A') \to Ext^2(Z, A)$ is induced by the map h': $A' \to A$ which is induced by the map h: $H' \to H$.

In particular, the characteristic class of the extension $1 \to H \to G \to \pi \to 1$ is equal to the characteristic class of the extension $1 \to A \to G/[H, H] \to \pi \to 1$. This latter extension has an abelian kernel A and so is characterized by a cohomology class[10] in $H^2(\pi, A)$. In §4, I will show that this cohomology class is, in fact, equal to the characteristic class u.

3. The reduction theorem. Let π be any group and j: $F \to \pi$ an epimorphism of a free group F onto π. Let R be the kernel of j and let $A = R/[R, R]$ be the factor commutator group of R. Let $u \in Ext^2_{\mathfrak{C}}(Z, A)$ be the characteristic class of the extension $1 \to R \to F \to \pi \to 1$.

REDUCTION THEOREM. *Let C be any $Z(\pi)$-module. Then the map $Ext^i_{\mathfrak{C}}(A, C) \to Ext^{i+2}_{\mathfrak{C}}(Z, C)$ by $\alpha \to u\alpha$ is an isomorphism for $i > 0$.*

Proof: Since F is free, we can find a free resolution for Z over $Z(F)$ of the form[11]

$$0 \to P_1 \to P_0 \to Z \to 0$$

Therefore, the characteristic class u is represented by the exact sequence

$$0 \to A \to P_1/R \to P_0/R \to Z \to 0$$

Since P_1 and P_0 are $Z(F)$-free, P_1/R and P_0/R are $Z(\pi)$-free. The theorem therefore follows from Lemma 1.

4. *Relations with cohomology.* If A and C are $Z(\pi)$-modules, we make *Hom* (A, C) a $Z(\pi)$-module by setting (xf) (a) = $xf(x^{-1}a)$ for $x \in \pi$, $a \in A$, $f \in Hom(A, C)$.

There is a natural map η: $Ext^n_\pi(A, C) \to H^n(\pi, Hom(A, C))$ defined as follows.[2] Let

$$E: \quad 0 \to C \to E_{n-1} \to \ldots \to E_0 \to A \to 0$$

represent $\alpha \in Ext^n(A, C)$. Since this sequence splits over Z, the sequence

$$E': \ 0 \to Hom(A, C) \to Hom(A, E_{n-1}) \to \ldots \to Hom(A, A) \to 0$$

is exact. Let

$$P: \quad \ldots \to P_n \to P_{n-1} \to \ldots \to P_0 \to Z \to 0$$

be a projective resolution of Z over $Z(\pi)$. Then[9] there is a map $f: P \to E'$ such that $f|Z: Z \to Hom(A, A)$ sends 1 into i, the identity map of A. Now $f_n: P_n \to Hom(A, C)$ is a cocycle in $Hom_\pi(P, Hom(A, C))$. We define $\eta(\alpha)$ to be the cohomology class represented by f_n. It is trivial to verify that η is well defined and natural.

LEMMA 2. *For all n, the map $\eta: Ext^n_\pi(A, C) \to H^n(\pi, Hom(A, C))$ is an isomorphism. Under this isomorphism, the Yoneda product corresponds to the cup product associated with the coefficient homomorphism $Hom(A, B) \otimes Hom(B, C) \to Hom(A, C)$ given by composition of mappings.*

Proof: The map η is clearly an isomorphism in dimension 0. If $n > 0$ and P is weakly projective, then $Ext^n_\pi(P, C) = 0$ for all C. Also, if P is weakly projective, then $Hom(P, C)$ is weakly injective[12] and so $H^n(\pi, Hom(P, C)) = 0$ for all $n > 0$. Consequently, the fact that η is an isomorphism for all n follows as in the standard uniqueness theorem[13] (using the Z-split epimorphism $Z(\pi) \otimes A \to A$ by $x \otimes a \to xa$). The cup products and Yoneda products behave in the same way with respect to connecting homomorphisms. Therefore, the argument used to prove the uniqueness theorem shows that if η preserves the products $Ext^i \otimes Ext^j \to Ext^{i+j}$, it also preserves the products $Ext^{i+1} \otimes Ext^j \to Ext^{i+j+1}$. By dualizing the argument used to prove the uniqueness theorem, using the weakly injective resolution $C \to Hom(Z(\pi), C)$ of C rather than a weakly projective resolution of A, we see that η also preserves the products $Ext^i \otimes Ext^{j+1} \to Ext^{i+j+1}$. Since η clearly preserves products in dimension 0, it must preserve all products.

LEMMA 3. *Let $1 \to A \to G \to \pi \to 1$ be a group extension with abelian kernel A. Let $u \in Ext^2(Z, A)$ be its characteristic class. Then $\eta(u) \in H^2(\pi, A)$ is the usual cohomology class associated with the group extension by taking factor sets.*[10]

Proof: Let

$$\ldots \to P_2 \to P_1 \to P_0 \to Z \to 0$$

be the standard nonhomogeneous complex[14] over $Z(G)$. Apply to this complex the construction of §2. Recall that the natural isomorphism (1) is obtained by sending $x \in A$ into the homology class in $H_1(A, Z)$ represented by the cycle $(x) \in P_1/H$. Now,

let

$$\ldots \to C_2 \to C_1 \to C_0 \to Z \to 0$$

be the standard nonhomogeneous complex over $Z(\pi)$. In order to compute $\eta(u)$, we must find a map

$$\begin{array}{ccccccccc} \ldots & \to & C_2 & \to & C_1 & \to & C_0 & \to Z \to & 0 \\ & & \downarrow f_2 & & \downarrow f_1 & & \downarrow f_0 & \downarrow = & \\ \ldots & \to & A & \to & X_1 & \to & P_0/A & \to Z \to & 0 \end{array}$$

It is trivial to verify that such a map is given by choosing a "cross section" k: $\pi \to G$ (not necessarily a homomorphism) such that $\pi \xrightarrow{k} G \to \pi$ is the identity and setting $f_0(\ ) = (\ )$, $f_1(x) = (kx)$, and $f_2(x, y) = a_{x,y}$ where $a_{k,y} = k(x)k(y)k(xy)^{-1}$. Thus $a_{x,y}$ is a cocycle representing $\eta(u)$. However, $a_{x,y}$ is just the factor set of the given extension.[10]

Using Lemmas 2 and 3, Theorem 1 can be restated as follows.

CUP PRODUCT REDUCTION THEOREM.[1] *Let $j: F \to \pi$ be an epimorphism of a free group F onto a group π. Let R be the kernel of j. Let $A = R/[R, R]$ be the factor commutator group of R. Let $v \in H^2(\pi, A)$ be the cohomology class associated with the extension $1 \to A \to F/[R, R] \to \pi \to 1$, the action of π on A being the usual action of a group on the kernel of an abelian extension. Let C be any $Z(\pi)$-module. Then the map $H^i(\pi, Hom(A, C)) \to H^{i+2}(\pi, C)$ by $\alpha \to v\alpha$ is an isomorphism for $i > 0$.*

[1] Eilenberg, S., and S. Mac Lane, "Cohomology theory in abstract groups *I*," *Ann. of Math.* **48**, 51–78 (1947).

[2] Yoneda, N., "On the homology theory of modules," *J. Fac. Sci. Univ. Tokyo*, **7**, 193–227 (1954). The theory in abstract categories is given by Buchsbaum, D. A., "A note on homology in categories," *Ann. of Math.*, **69**, 66–74 (1959).

[3] Hochschild, G., "Relative homological algebra," *Trans. Amer. Math. Soc.*, **82**, 246–269 (1956).

[4] In the terminology of Buchsbaum, *op. cit.*, the selected class 3 of momomorphisms consists of those whose image is a Z-direct summand of their range.

[5] Buchsbaum, D. A., *op. cit.*, Prop. 2.2.

[6] Hochschild, G., *op. cit.*

[7] Buchsbaum, D. A., *op. cit.*, Th. 3.1.

[8] Cartan, H., and S. Eilenberg, *Homological Algebra* (Princeton: Princeton Univ. Press, 1956), chap. 10, §4.

[9] *Ibid.*, chap. 5, Prop. 1.1.

[10] *Ibid.*, chap. 14, §4.

[11] *Ibid.*, chap. 10, §5.

[12] *Ibid.*, chap. 10, Prop. 8.5.

[13] *Ibid.*, chap. 3, Prop. 5.2.

[14] *Ibid.*, Chap. 9, §6 and Chap. 10, §4.

*MECHANICAL GERMINATION OF BACTERIAL SPORES**

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Webster's New International Dictionary[1] defines germination as (the) "Process of germinating; beginning of vegetative growth; as: BOT. Beginning of growth or development of a spore." This definition is not very helpful because it does not allude to the essential incipiency of the process which results in a change in the state of the ungerminated cell to the germinated cell. Clearly, from a theoretical standpoint, this is by far the most interesting and important aspect of germination. However, it is still the most mysterious aspect of germination. The definition also implies that germination is strictly a physiological or enzymatic process.

Although the germination of bacterial spores has been studied intensively in recent years, the prime event in germination still has escaped detection. It is, nevertheless, possible operationally to characterize germination immediately after the prime event takes place. Phenomena such as nuclear division, cell wall synthesis, cellular elongation, and cellular division clearly are secondary and, accordingly, are irrelevant to the germination process. In a recent review of the criteria of spore germination, Campbell[2] includes all the secondary changes in the generic term "outgrowth."

Germination of a bacterial spore is an all or nothing proposition, and with current technology it is now possible to assert a number of specific features which constitute a *sine qua non* for the earliest and, therefore, minimal condition of germination. The acquisition of these features goes hand in hand with the germination process and, indeed, must be regarded as collectively constituting the germination process itself.

Germination of bacterial spores has heretofore been obtainable only in a suitable nutritional environment at an appropriate pH, oxidation-reduction potential, oxygen tension, temperature, and so fourth. The following features are invariably associated with the physiological germination of each bacterial spore: (*a*) colony-forming ability in suitable growth medium, (*b*) loss of viability upon exposure to environmental stresses which do not affect the viability of the ungerminated spore (e.g., temperature, desiccation, ultraviolet and x-ray radiation, bactericidal chemicals, etc.),[3] (*c*) loss of refractility of the spore cell with simultaneous darkening when examined by phase contrast microscopy,[4] (*d*) marked reduction in the optical density to visible light of a suspension of bacterial spores in water,[5] (*e*) an apparent slight enlargement of the cell as though it were somewhat swollen, (*f*) stainability by methylene blue or crystal violet,[5] (*g*) a decrease by about one-third in the dry weight of the ungerminated spore,[6] (*h*) the elimination into the aqueous environment, in soluble form, of practically all of the dipicolinic acid, a large part of the calcium and a nondialyzable polypeptide,[6] and finally, (*i*) increased respiratory activity at the expense of glucose.[7]

On the basis of present knowledge, it seems safe to say that in the genus Bacillus spore germination is characterized by each and every one of the above features. Conversely, where each and every one of the above features can be shown to apply, it follows that the process of germination has taken place.

For the past two years we have been studying dipicolinic acid in bacterial spores, in *Bacillus megaterium* in particular. Our results indicate that in ungerminated spores the dipicolinic acid exists unconjugated by the usual primary valence bonds and that it is released in the free (metal chelate) form under conditions where enzymatic action is precluded. More important are the numerous results in which the release of dipicolinic acid was induced by a variety of different treatments without an apparent loss of the structural integrity of the spore cell.[8] We have construed these results to mean that the metal chelate of dipicolinic acid is disposed superficially in the spore in such a manner as to be extremely liable to induced release from the spore. An extensive study of the release of dipicolinic acid by surface active agents at slightly elevated temperatures[9] revealed that the release was accompanied by most of the criteria listed above. Were it not for the fact that the treated cells failed to form colonies, chemical germination[10] of the spores would have been achieved. In all probability, chemical germination actually was achieved, the loss of viability having been shown to be a secondary consequence of the hypersensitivity of the germinated cell to the concentration of surfactant required to effect germination.

Physical methods for nonphysiological germination of bacterial spores have also been investigated, with promise of success in a procedure employing electrodialysis.[8] The present paper is concerned with a purely mechanical approach to nonphysiological germination of bacterial spores, namely, the nonlethal abrasion of bacterial spores by agitation with ground glass.

Production of spores: Spores of *Bacillus megaterium* were produced on the surface of 2 per cent agar medium in petri dishes for 3 days at 30°. The production medium was that employed by Slepecky and Foster[11] except that glucose was substituted for sucrose and 0.1 per cent L-glutamic acid and 0.05 per cent Basamin (Anheuser-Busch) were added. The spores were harvested, washed 6 times with sterile deionized water, and stored in water at 4°. Such suspensions consisted entirely of refractile spores and remained unchanged under these conditions of storage.

Recovery medium and inoculation procedure: Two per cent nutrient agar (Difco) supplemented with 0.1 per cent soluble starch was employed to determine the ability of viable cells to form colonies. For the plate counting, 0.1 ml to 0.3 ml of suitable dilutions of cells was spread with sterile glass rods uniformly over the surface of the agar in petri dishes. Quintuplicate plates were prepared in every case. The colonies were counted after incubation for 16 to 24 hours at 37°.

Preparation of the ground glass: Glass homogenizing beads,[12] approximately 200 ± 30 μ in diameter, were passed through an impact type micro pulverizer. The fraction which passed through a Tyler Standard Screen[13] (325 mesh; 44 μ openings) was collected and cleaned with hot, concentrated hydrochloric acid, followed by exhaustive washing with deionized water, and dried in an oven at 100°. It consisted of jagged particles of glass ranging from 44 μ to subspore size.

Mechanical abrasion in the Mickle tissue disintegrator: The Mickle tube was loaded with 4 gm of the ground glass and 7 ml of a cold 1:5 dilution of the spore stock (approximately 7×10^{10} spores; 13 mg dry weight) in M/15 phosphate buffer, pH 7.0. The spores were exposed to the abrasive effect of the ground glass by shaking in the Mickle disintegrator at 4° for the desired periods of time.

Chemical procedures: Samples contained in glass-sealed ampules were hydro-

lyzed in 6 *N* HCl at 106° for 15 hours. Following evaporative removal of the HCl, amino acids were estimated as L-alanine equivalents.[14] Total protein was determined according to the method of Lowry, *et al.*,[15] and hexosamine by a modification[16] of the Elson and Morgan procedure. Extraction, spectrophotometric quantitation and characterization of dipicolinic acid were performed by methods described previously.[17, 18]

Conversion of spores to heat sensitive cells by shaking with glass particles: Spores were agitated in the Mickle instrument with the ground glass for the periods of time shown in Table 1. A separate aliquot was agitated for each of the time intervals. After the indicated times, the glass was allowed to sediment for 1 to 2 minutes and 0.5 ml of the turbid supernatant liquid was transferred to a tube containing 9.5 ml of M/15 phosphate buffer, pH 7.0. Three subsamples from this tube were separately diluted further in buffer to final dilutions of 10^6 to 10^7. The number of viable cells in each of the final dilution tubes was determined by the spread plate procedure both before and after heating of the dilution tubes at 65° for 20 minutes. All dilutions were prepared with cold buffer and were held in an ice bath. The recovery plates, after incubation, had a minimum of 31 to a maximum of 309 colonies. The dilution tube from which the final plating was done was also the tube which was heated. The highest viable cell count of a heated dilution tube was 2780 cells per ml and the lowest was 840 cells per ml. Excellent agreement was obtained not only among the 3 subsamples but also among the replicate plates for each of the time periods.

Two controls were run in this experiment. For one, a 7 ml aliquot of the spore suspension was shaken without the ground glass for 24 minutes in the Mickle disintegrator, the conditions being otherwise identical with the ground glass treatments (Control I). For the second control, an extra spore aliquot was given a 24-minute shaking exposure to ground glass after which the particulate matter was removed by centrifugation at 6,000 × *g*. The supernatant liquid (i.e., spore extract) was then used to suspend normal spores, which were then shaken without ground glass for 24 minutes in the Mickle instrument. The suspension was then plated for counting before and after heating at 65° for 20 minutes (Control II). Control II was designed to exclude the possibility that germination of normal spores, consequent to the exposure to soluble materials derived from ruptured spores, might occur during the process of abrasion.

The results of this experiment (Table 1) show that the original suspension consisted entirely of spores which withstood the heat test. This was true also of spores which had been shaken for 24 minutes in the absence of ground glass, whether suspended in M/15 phosphate buffer (Control I) or suspended in the "spore extract" (Control II). On the other hand, in the suspension shaken with the ground glass the number of viable cells decreased as the abrasion progressed. However, of the viable cells remaining, a sizable fraction was now killed by the heat treatment where none was killed before the abrasion. After 12 to 24 minutes abrasion, approximately 80 to 90 per cent of all the spores which had survived the abrasion process and which were recoverable as colonies in plates were no longer heat resistant. As many as 45 per cent of all the spores in the starting suspension were converted from heat resistant to heat sensitive cells by the abrasion treatment (12 minutes). Although we refer to the effect of shaking in the presence of the glass particles as an

TABLE 1

SENSITIVITY TO HEAT* INDUCED IN SPORES OF *Bacillus megaterium* BY ABRASION WITH GLASS PARTICLES

	Abrasion Time Minutes	Per Cent of the Spores Which Survived the Abrasion Total	Heat Sensitive	Per Cent of the Survivors Which Are Heat Sensitive	Per Cent of Cellular Units Not Refractile‡
	0	100	0	0	0
	3	50	10	20	10
	6	52	22	42	20
	9	55	33	59	42
	12	58	45	78	51
	15	36	32	88	70
	18	27	24	86	82
	24	23	21	89	84
Control I	24 minutes, no glass	95	0	0	0
Control II	24 minutes, no glass; spores suspended in spore extract†	105	0	0	0

* Killed in 20 minutes at 65°.
† See text.
‡ Includes nonviable entities (ghosts?).

TABLE 2

LETHAL EFFECTS OF VARIOUS TREATMENTS ON ABRADED SPORES

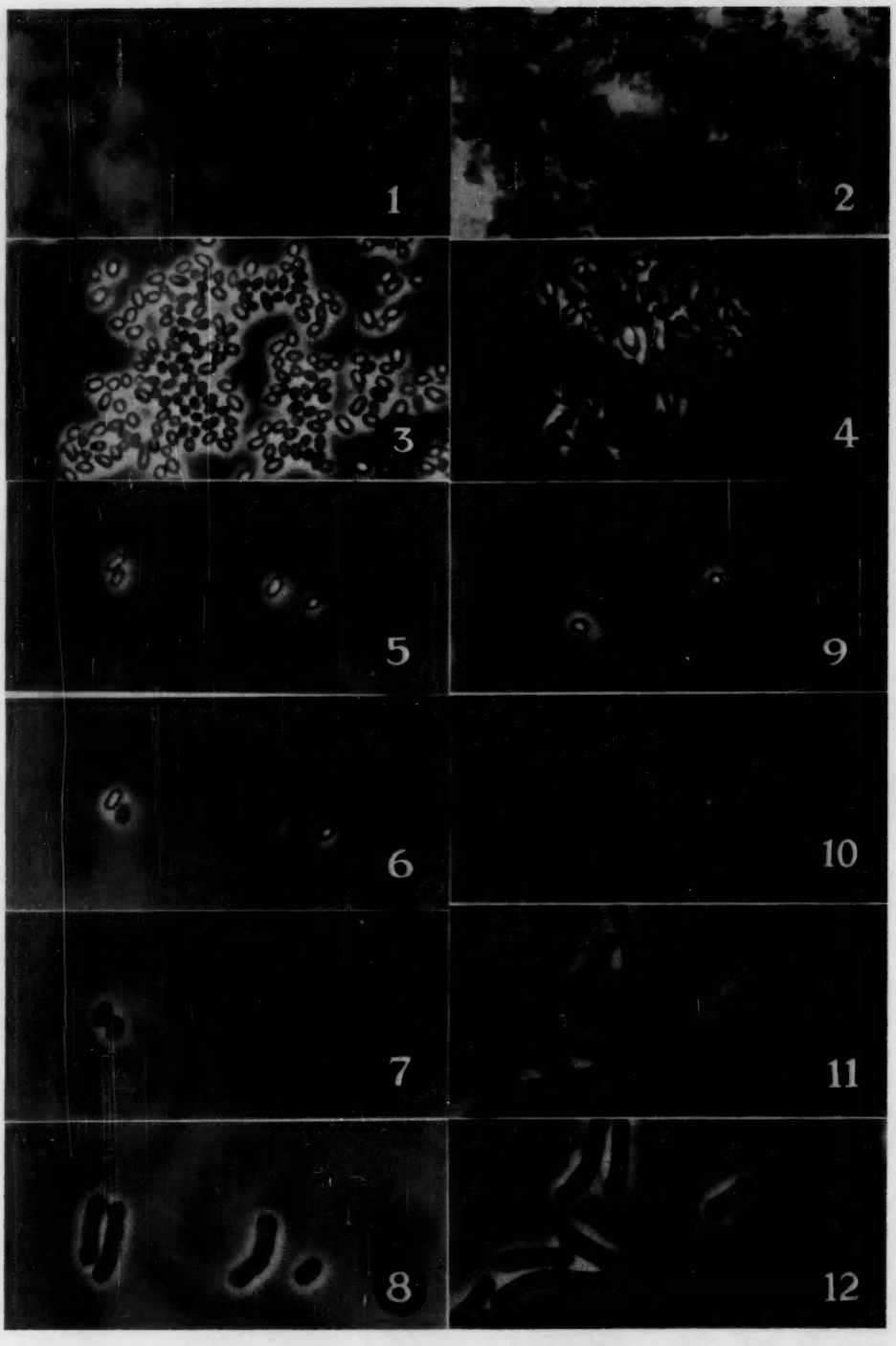
Treatment	Concentration or Dosage	Time of Exposure and Temperature	Per Cent of Spores Killed Control (Unabraded)	Abraded Spores*
None			0	0
Heat		20 min., 65°C	0.5	75
Ethanol	80 per cent	10 min., 4°C	29	63
H_2O_2	15 per cent	10 min., 4°C	23	66
Phenol	3 per cent	10 min., 4°C	8	19
Ultraviolet irradiation	4,800 ergs/mm²	80 sec., 25°C	16	49
Freeze-Thaw	8 times	−80°C; +37°C	28	63

* An aliquot of the same suspension of spores used in the unabraded control spores was abraded for 12 minutes with the special glass fraction in the Mickle tissue disintegrator at 4°C.

abrasion, it is possible that some of the effects are achieved by direct "cracking" of the spore. Clarification of this point is expected from thin-section microscopy studies which are under way.

Spores of *B. megaterium*, abraded as described in the foregoing, were also more sensitive to the lethal effects of other treatments known to be more effective against vegetative cells than against ungerminated spores (Table 2). In evaluating these data the necessary assumption is made that the various treatments employed did not cause more clumping among spores that had been abraded than among the control spores. This was not verified but, on the other hand, neither is there any reason to believe clumping was greater.

Microscopic study of abraded spores: The normal, ungerminated spores of *B. megaterium* are highly refractile (Figure 3) and are not stained by the usual vegetative cell stains (Figure 1). As a consequence of the abrasion with glass particles, a sizable fraction of the refractile, nonstaining spore population became nonrefractile (Figure 4), and also stainable (Figure 2). The proportion of nonrefractile, stainable cells increased with the length of the abrasion period (Table 1). Mechanical disruption of bacterial spores has been described several times. In some cases,[6, 19, 20] stainability and loss of refractility occurred, and in other cases certain other changes.[21]



FIGS. 1–12.—Photomicrographs of *B. megaterium*, magnification ×1,666.

Figures 1 and 2 show fixed films stained 2 minutes with gentian violet and photographed through an ordinary light microscope. Figure 1 shows that normal, ungerminated spores do not

Having established that heat sensitive cells were produced during the abrasion treatment, as shown by the colony-forming potential of the cells, it next became essential to demonstrate directly that the dark, nonrefractile cellular fraction of the abraded suspension was viable. This was undertaken by microscopic inspection and time lapse photomicrography recording the outgrowth of such cells.

As a basis for comparison, the germination and out-growth of unabraded spores was included. Normal, refractile spores of *B. megaterium* were introduced suitably on a film of nutrient agar on a glass microscope slide and a cover slip applied. Provision was made for retardation of evaporation and for availability of oxygen. The living preparation was viewed under dark contrast phase illumination and the sequence of events recorded in Figures 5 through 8. The typical pattern of initial loss of refractility and darkening was obser[illegible] (Figures 6 and 7). This was followed by swelling, increase in size, and elong[illegible]on to the characteristic rod-shaped vegetative cells of this species (Figure 8). Under the conditions employed, at least 30 minutes was required for changes in refractility of significant numbers of normal spores.

Figures 9 through 12 show a time lapse sequence of one field of cells obtained from an abraded suspension. Figure 9 contains 3 refractile, normal, ungerminated spores and 6 abraded spores and was photographed within 5 minutes after addition of nutrients, well before germination of refractile spores occurred. Also, the picture is the same in the absence of added nutrients; consequently, the dark, nonrefractile bodies were the products of the mechanical abrasion and were not physiologically germinated spores. Figure 10 shows that 2 of the normal spores germinated (loss of refractility and darkening). The figure also shows that 4 of the abraded cells had swelled and definitely commenced growth and elongation. These processes are extended in Figures 11 and 12. This series of photographs reveals (*a*) that the abraded spores are viable and capable of outgrowth to typical vegetative cells, (*b*) that by these microscopic procedures abraded spores are indistingushiable from the cells produced from normal spores by physiological germination, and (*c*) that the outgrowths of both kinds of cells are undistinguishable. Expressed differently, abraded spores behave like physiologically germinated spores.

stain. Figure 2 shows that many spores in a suspension that had been abraded with ground glass are stainable.

Figures 3 and 4 show living spores in wet mount preparations photographed through a dark contrast phase microscope. Figure 3 shows the highly refractile nature of normal, ungerminated spores. Figure 4 shows an abraded spore suspension containing some normal, refractile spores and many nonrefractile, dark, abraded spores. No stain was applied to these preparations.

Figures 5, 6, 7, and 8 show a time lapse series of the same field of living spores on a nutrient agar film, photographed through a dark contrast phase microscope. Figure 5 shows 4 normal, ungerminated spores. After 70 minutes, 2 of the spores have germinated as shown by loss of refractility and darkening (Fig. 6), and after 115 minutes all of the spores had become nonrefractile and dark, and slightly swollen (Fig. 7). Figure 8 shows the elongation and outgrowth to vegetative cells.

Figures 9, 10, 11, and 12 show a time lapse series of the same field of living spores of a suspension that had been abraded with ground glass. The cells were on a nutrient agar film, photographed through a dark contrast phase microscope. Figure 9 shows the field contained at the start 3 normal, ungerminated, refractile spores and 6 nonrefractile, dark, abraded spores. Figure 10, the same field 2 hours later. Two of the ungerminated spores have germinated, losing their refractility and becoming dark. Four of the abraded cells are swollen and are beginning to elongate. Figure 11, the same field 30 minutes later. The last ungerminated spore has germinated and the abraded cells have elongated further. Figure 12, the same field 40 minutes later, showing that the vegetative cells derived from abraded cells are indistinguishable from those derived from physiologically germinated cells.

Spore contents released during abrasion: During physiological germination of spores of *Bacillus megaterium*, about 30 per cent of the dry weight of the spore is excreted into the medium.[6] More than half of the excreted solids consisted of calcium dipicolinate, 10 to 15 per cent of a hexosamine-containing nondialyzable peptide (molecular weight approximately 10,000),[22] and the rest a mixture of small amounts of protein, free amino acids and unidentified low molecular weight peptides. In an attempt to extend further the similarity of the changes in spores induced by mechanical abrasion and by physiological germination, the water-soluble fraction produced during abrasion was analyzed for dipicolinic acid, protein, amino acids and hydrolyzable amino acids and hexosamine.

The mixture of spore suspension and glass particles of each of the 8 preparations described in Table 1 was diluted to 26.5 ml with M/15 cold phosphate buffer, centrifuged and the clear supernatant liquid used for the analyses.

Without presenting the analytical data, it may be stated that during the abrasion process there was a progressive increase in the amount of dipicolinic acid, protein, amino acids, and hydrolyzable amino acids and hexosamine released in soluble form. In 24 minutes the amounts released represented a major proportion of the maximal amount of these constituents which is releasable by physiological germination. The data cannot be taken at face value, for the recovery of viable cells and the microscopic evidence of debris and "ghosts" indicate that some of the cells were completely disrupted with consequent release of soluble constituents. Also, attempts to find a systematic quantitative relation between the total cells, heat sensitive cells and constituents released were unsuccessful (see below). Nevertheless, the bulk of the materials released were of the type that are released during physiological germination.

One of the main problems in the quantitation is caused by the clumping and/or trapping of some of the spores by the sedimenting glass particles after the abrasion process. Thus, the values for the survivors given in Table 1 have been shown to be low, especially for the shorter periods of abrasion (see footnote to Table 3).

TABLE 3

DIPICOLINIC ACID RELEASE FROM ABRADED SPORES AT 65°

Abrasion Time, Minutes	Exposure after Abrasion for 20 Minutes at 4°		Exposure after Abrasion for 20 Minutes at 65°	
	Survivors,* Per Cent	Cell-Bound DPA, Per Cent	Survivors,* Per Cent	Cell-Bound DPA, Per Cent
0	100	100	100	98.8
3	94	90	73	65
12	62	59	18	15

* Corrected values based on the fraction of total dipicolinic acid recovered in the supernatant suspension after settling out of the glass particles. The fractions were 34 and 77 per cent for the 3 and 12 minute periods, respectively.

Study of this matter revealed the interesting fact that the great majority of the abraded cells may under some conditions retain their dipicolinic acid. The dipicolinate was found to be released at 65°, a temperature which is totally ineffective in releasing dipicolinic acid from normal ungerminated and unabraded spores (Table 3). The simplest explanation of these results is that under the conditions employed (4° and rapid handling) a fraction of the spores whose permeability barrier was breached by the abrasion had not been rapidly penetrated by water to dissolve and remove the dipicolinic acid. Mild heating for a short time markedly accelerated the release. An alternative explanation would be that the dipico-

linic acid was released from a chemically bound form. This explanation is not favored because of the lack of evidence for a bound form of dipicolinic acid in this organism.[8]

Respiratory activity of abraded spores: Figure 13 shows that normal spores, either unheated or heated 20 minutes at 65°, did not oxidize glucose. On the other hand, a marked uptake of oxygen was observed with unheated and heated spores after they had been abraded. The respiratory activity of the abraded spores was eliminated by subsequent heating at 65° for 20 minutes.

Figure 14 reveals that the respiratory activity was confined to the insoluble, centrifugable fraction containing unaltered spores, abraded spores and cellular debris. The activity was probably not attributable to germination of residual,

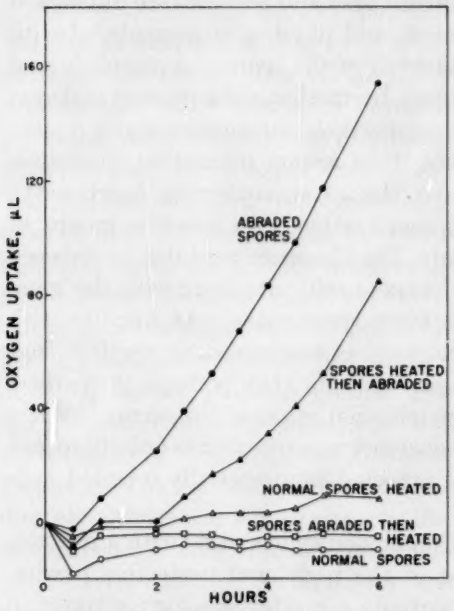


FIG. 13.—Oxidation of glucose by various types of spore suspensions. The suspension fluid was *M*/15 phosphate buffer, pH 7.0, temperature 30°C. The abraded spores were prepared by agitation with glass particles in a Mickle disintegrator for 24 minutes at 4°C. Where heating is designated the conditions were 20 minutes at 65°C.

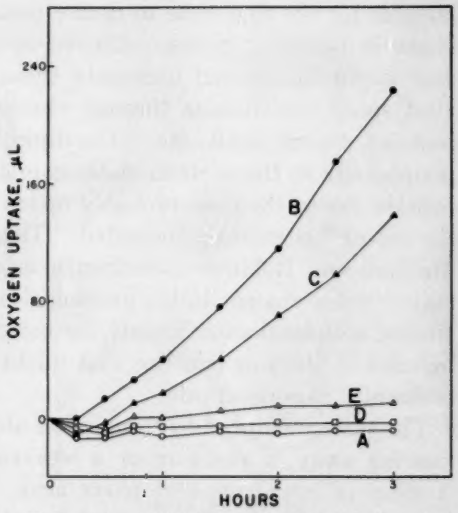


FIG. 14.—Oxidation of glucose by (*A*) normal spores, (*B*) abraded spores, (*C*) insoluble fraction of abraded spores, (*D*) supernatant liquid from abraded spores, (*E*) normal spores in supernatant liquid from abraded spores. Spore suspensions were made in *M*/15 phosphate buffer, pH 7.0. Temperature 30°C.

normal spores in the soluble spore components released during the abrasion process since a suspension of normal spores, when placed in an identical supernatant liquid from abraded spores, did not oxidize glucose in the course of the experiment.

It is evident that abraded spores displayed clear-cut respiratory activity under conditions where normal spores were for all practical purposes inert. This respiratory activity was heat sensitive. However, the same heat treatment applied to the spores before abrasion was ineffective in suppressing the respiratory activity of the subsequently abraded cells, except for a delay. The protective mechanism in normal spores was absent in abraded spores.

Discussion.—In every respect that has been considered experimentally, the

changes in the spores induced by the abrasion with glass particles are indistinguishable from those induced by physiological germination. Each of the available criteria listed in the introductory part of this paper, with the exception of (*d*) which was not studied, and which characterize germination under physiological conditions, is also applicable to the abraded spores. The most reasonable conclusion is, therefore, that the abrasion process results in mechanically germinated spores. Conditions have been worked out whereby a very substantial proportion of the starting spore suspension was germinated mechanically, although it is quite possible that the efficiency could be increased appreciably. Techniques of differential centrifugation permit the obtaining of fractions enriched in the mechanically germinated spores and these are being studied further.

The surprising readiness with which dipicolinic acid and peptides are discharged from spores by a variety of thermal, chemical, and physical treatments,[8, 9] with no apparent destruction of the structural integrity of the spore as a morphological entity, now culminating in a comparable effect by mechanical abrasion, makes it possible for the first time to derive certain supportable conclusions about dipicolinate in bacterial spores. Almost certainly these results mean that dipicolinic acid is not distributed uniformly throughout the spore, conferring resistance to vital spore constituents through chemical combination with reactive groups in proteins, nucleic acids, etc. The dipicolinate, like the spore peptides, is disposed peripherally to the central, viable core of the spore cell. It, along with the spore peptide, layers the core, probably under the outer spore coats. An identity with the cortex[23] is strongly indicated. This suspicion is heightened by the fact that Mayhall and Robinow's electron micrographs indicate that perhaps the earliest major visible change during physiological germination is loss of the cortex. When studied with lanthanum nitrate, the cortex substance was revealed as orderly spaced bundles of fibers or lamellae that might be expected from spatially oriented molecules of the spore peptide.

The change induced by mechanical abrasion obviously has to do with a physical wearing away, a cracking or a weakening of the peripheral protective barrier. A series of interdependent phenomena, essentially a matter of allowing water to enter and to dissolve away the calcium dipicolinate and spore peptides, then follow. As Powell[24] put it, ". . . the structure of this integument is of primary importance for the maintenance of the heat stable structure of the resting spore." The cardinal function of the protective barrier now emerges more clearly than ever to be one of preventing water from reaching the core of the spore. This impermeable waterproofed barrier may be breached by chemical means (surfactants, H_2O_2, etc.,[8, 9] ethylene diamine tetracetate[25]), by mechanical abrasion, or by physiological germinants.[10] Although the latter are usually interpreted on an enzymatic basis, it is, nevertheless, possible that they function as specific chemical agents capable of nonenzymatic reaction with surface components of the spore making up the outermost permeability barrier. The ingress of water results in entrance of stains, hydration of the cytoplasm with resultant reduction in refractive index, and the display of metabolic activity of preexisting enzymes.

In the final analysis, to reiterate what has been proposed on several occasions by previous investigators,[24, 26] the unique resistance of the bacterial spore is attributable to its anhydrous state. The remarkable extent to which this state can confer

tolerance to the otherwise lethal effects of heat are illustrated in Figure 15, which records an experiment dealing with the viability of vegetative cells of *B. megaterium*. Effects of such magnitude afford strong support for the anhydrous state of the spore accounting for its unique resistance properties.

The intricacies of the germination and hydration of the spore are now well on their way to being exposed. It is already apparent that the problem of hydration has given way to the problem of anhydration as the next major feature unique to the bacterial spore. To achieve the remarkable degree of anhydration in an aqueous environment, the spore-forming cell obviously must invoke an equally remarkable dehydrating mechanism, probably chemical. It is here that the role of the metal chelates of dipicolinic acid may well lie.

Summary.—A suspension of spores of *Bacillus megaterium* and ground glass particles was shaken in a Mickle tissue disintegrator under conditions in which physiological germination was precluded. Approximately 80 to 90 per cent of the viable, colony-forming cells remaining after 12 to 24 minutes shaking were heat sensitive, being killed in 20 minutes at 65°. These "abraded" spores were nonrefractile, were stainable with bacteriological stains, were sensitive to a number of lethal agents, oxidized glucose and did not retain their dipicolinic acid and spore peptides. Time lapse photomicrographs showed the outgrowth of individual abraded spores. In all respects, the abraded spores were similar to physiologically germinated spores and they are regarded as mechanically germinated spores. The mechanism of mechanical germination is discussed in terms of a general theory of spore resistance based on the anhydrous state of the spores and its protection by a permeability barrier. Breaching the permeability barrier constitutes the prime event in germination. A striking tolerance to heat by lyophilized (anhydrous) vegetative cells of *B. megaterium*, as shown by quantitative viability counts, is presented in support of the anhydration theory.

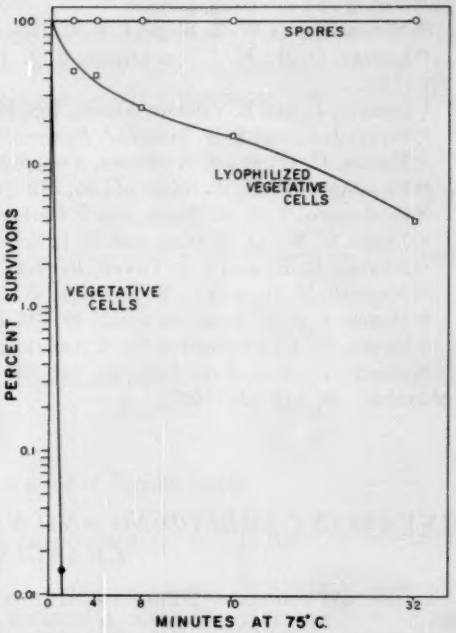


FIG. 15.—Curves showing the survival rates of lyophilized vegetative cells of *Bacillus megaterium* when heated at 75°, compared to the corresponding curves for nonlyophilized vegetative cells and for spores.

* This investigation was supported in part by the Microbiology Branch of the Office of Naval Research.

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INVARIANT IMBEDDING AND NEUTRON TRANSPORT IN A ROD OF CHANGING LENGTH

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In a recent series of papers[1–6] the authors have used the method of invariant imbedding, an extension of the invariance principles of Chandrasekhar and Ambarzumian, to study neutron behavior in fixed media. Here we consider a case in which the region changes in a specified manner as the neutron process goes on. We shall be content at present merely to formulate the equations for a simple problem. Existence and uniqueness of the solution will be established in a subsequent paper. It is hoped that eventually cases in which the change in the medium actually depends upon the neutron process itself may be handled by similar devices, so that an attack upon Stefan-type problems using invariance principles will be possible.

Our model is the simple one of neutrons moving in a one-dimensional rod, the process being initiated by a trigger neutron impinging on the right end of the rod. The length of the rod changes with time, the variation occurring at the left end. We investigate the reflected flux—the expected number of neutrons emerging from the right end of the rod as a function of time.

The Physical Process and Its Mathematical Formulation.—A rod extends from 0 to x at time $t = 0$. The rod grows or erodes at a specified rate so that the position of the left end is given by $X_L = f(t), f(0) = 0$. The right end remains fixed, $X_R = x$. A neutron traversing a distance Δ in the rod has probability $\sigma\Delta + o(\Delta)$ of suffering a collision in the medium. In the event of a collision, two neutrons emerge, one moving to the left, the other to the right. No neutron can re-enter the rod once having left it, and all neutrons have constant velocity v. A single neutron, the "trigger," enters the rod at x at time $t = s$. We ask for the expected total number that emerge at the right end up to time t, denoting this quantity by $U(x, s, t)$.

It must be noted that the condition $(-f'(t)) < v$ for all t is necessary to prevent neutrons which have emerged from the rod at x_L from being overtaken by the end of the rod and reentering the system.

To apply the invariant imbedding method, we imbed the original process within the class of all processes indexed by $x > 0$, and then express the relationship between neighboring processes. The left ends are to obey the same law, $x_L = f(t)$, as that of the original rod.

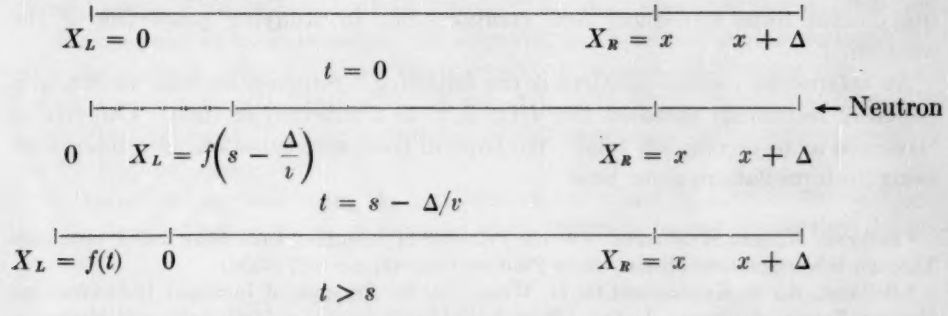

FIG. 1.—Neutron transport in a rod of varying length.

At time $t = s - \frac{\Delta}{v}$ the trigger neutron enters at $x + \Delta$. It may or may not suffer a collision in passing from $x + \Delta$ to x. If it does, a single neutron immediately emerges at the right. In either event, a neutron passes X_R at time $t = s$. This acts as a trigger for the original rod, and produces a flux emergent from X_R at times $t > s$. The expected rate of emergence of such neutrons is given by $u(x, s, t) = \partial U(x, s, t)/\partial t$. Neutrons in this flux may or may not suffer collisions with the rod material in going from x to $x + \Delta$. In either event $U(x, s, t)$ neutrons emerge from $x + \Delta$ by time $t + \Delta/v$. In addition, those neutrons which do make collisions in $(x, x + \Delta)$ at time t', $s < t' < t + \frac{\Delta}{v}$, contribute trigger neutrons at X_R at a rate $\sigma\Delta u(x, s, t') + o(\Delta)$. The resultant flux out of $x + \Delta$ up to time $t + \Delta/v$ is then $\sigma\Delta \int_s^t u(x, s, t')\, U(x, t', t)dt' + o(\Delta)$. All other processes yield contributions of order $o(\Delta)$.

Thus we find

$$U(x + \frac{\Delta}{v}, s - \frac{\Delta}{v}, t + \Delta) = \sigma\Delta + U(x, s, t) + \sigma\Delta \int_s^t u(x, s, t')\, U(x, t', t)dt' + o(\Delta). \tag{2.1}$$

Hence, letting $\Delta \to 0$,

$$\frac{\partial U}{\partial x} - \frac{1}{v}\frac{\partial U}{\partial s} + \frac{1}{v}\frac{\partial U}{\partial t} = \sigma + \sigma \int_s^t u(x, s, t')\, U(x, t', t)dt'. \tag{2.2}$$

The conditions imposed on U are

$$U(x, s, t) = 0, s \geq t;$$
$$U(x, s, t) = 0, f(s) = X_R. \tag{2.3}$$

The first merely states that no neutrons emerge by time t if the trigger enters at a later time, while the second reflects the fact that none can emerge to the right if the rod is of length zero when the trigger neutron impinges.

Concluding Remarks.—Equation (2.2) has the same general structure as that studied in our earlier papers[3, 6] for the case of the rod of fixed length with the neutron entering at $t = 0$. It is not difficult to reduce (2.2) to the simpler equation when $f(t) = 0$. However, the fact that the integral term in (2.2) is no longer of convolution form introduces new complications in studying properties of the solution.

An interesting control problem is the following. Suppose we wish to obtain a (suitably restricted) specified flux $U(x, 0, t)$ as a function of time. Can $f(t)$ be chosen so as to accomplish this? We hope to treat such questions at a later date, using the formulations given here.

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HYDROMAGNETIC WAVES IN A COMPRESSIBLE FLUID CONDUCTOR

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1. Introduction.—Consider an infinite mass of an electrically conducting fluid at rest, penetrated by a uniform magnetic field $\mathbf{H}_0$. Assume that as a result of a perturbation, a velocity field $\mathbf{v}$ is produced in a certain region, and that the magnetic field becomes $\mathbf{H}_0 + \mathbf{h}$. Alfvén[1] has demonstrated the possibility of propagation of $\mathbf{v}$ and $\mathbf{h}$ along the lines of force of the undisturbed magnetic field, in opposite directions, with velocities $a = \pm H_0 \sqrt{\mu_0/4\pi\rho_0}$, where μ_0 and ρ_0 are the permeability and the density of the medium which is assumed to be incompressible.

The theory does not apply to a compressible medium conductor. Compressibility effects have been considered by several authors; a current bibliography on the subject is given in Sears' survey article.[2] We shall approach the problem from a different point of view, however, concentrating our attention on the vorticity field and the current density. Once the vorticity field and the current density are found, the complete determination of $\mathbf{v}$ and $\mathbf{h}$ in their terms can be effected by available formulas. To take compressibility into account an important result of Jacob[3] may be used.

We note that in considering the propagation described by Alfvén, the velocity $\boldsymbol{v}$ and the magnetic field $\mathbf{h}$ can be replaced by the vorticity ω and the current density $\mathbf{j}$, where

$$\omega = (1/2) \operatorname{curl} \mathbf{v},$$

$$\mathbf{j} = (1/4\pi) \operatorname{curl} \mathbf{h}.$$

In taking this view as a point of departure, we shall show that, *in the case of a compressible medium, the components of* ω *and* $\mathbf{j}$ *in the direction of the field only, can be propagated in Alfvén's manner.* We shall give equations for the other components, but in view of applications of physical interest we shall subsequently assume that the components of vorticity perpendicular to $\mathbf{H}_0$ vanish. We shall conclude this note with some remarks about the wave equation and filter principles, following the researches of Brillouin and Parodi.[4] It appears possible to demonstrate some principal features of such propagation by means of simple mechanical systems similar to those studied by Ingard[5] for the case of an incompressible medium.

2. Fundamental Equations.—If, in order to simplify the discussion the dissipative effects are neglected and the Oz axis is taken in the direction of the field $\mathbf{H}_0$, the equations giving the variations in $\mathbf{v}$, $\mathbf{h}$ and ρ are

$$\rho_0 \frac{\partial \mathbf{v}}{\partial t} = - \operatorname{grad} \phi + \frac{\mu_0 H_0}{4\pi} \frac{\partial \mathbf{h}}{\partial z}, \tag{1}$$

$$\frac{\partial \mathbf{h}}{\partial t} = H_0 \frac{\partial \mathbf{v}}{\partial z} - \mathbf{H}_0 \operatorname{div} \mathbf{v}, \tag{2}$$

$$\frac{\partial \rho}{\partial t} + \rho_0 \operatorname{div} \mathbf{v} = 0, \qquad \operatorname{div} \mathbf{h} = 0, \tag{3), (4}$$

where we have set $\phi = p + \mu_0(\mathbf{H}_0 \cdot \mathbf{h})/4\pi + \rho_0\psi$, where ψ is the potential of external forces, which is assumed to be a solution of Laplace's equation, and p and ρ are the perturbations in pressure and density. The electromagnetic variables are taken to be measured in emu. Furthermore, it is assumed that

$$p = c^2\rho, \tag{5}$$

where c is the speed of sound in the fluid in the absence of a magnetic field.

Taking the curl of terms of equations (1) and (2) we obtain

$$2\rho_0 \frac{\partial \omega}{\partial t} = \mu_0 H_0 \frac{\partial \mathbf{j}}{\partial z}, \tag{6}$$

$$4\pi \frac{\partial \mathbf{j}}{\partial t} = 2H_0 \frac{\partial \omega}{\partial z} + \mathbf{H}_0 \times \operatorname{grad} \operatorname{div} \mathbf{v}, \tag{7}$$

which imply important consequences, as will be shown.

3. A Special Case of Propagation.—In the case when ρ does not depend on x and y, ω and j can be propagated along the lines of force of the undisturbed magnetic field with velocities $\pm a$. For in this case, by virtue of equation (3), equation (7) reduces to

$$2\pi \frac{\partial \mathbf{j}}{\partial t} = H_0 \frac{\partial \omega}{\partial z}. \tag{8}$$

Combining equations (6) and (8) we obtain

$$\frac{\partial^2 \omega}{\partial t^2} = a^2 \frac{\partial^2 \omega}{\partial z^2}, \tag{9}$$

and

$$\frac{\partial^2 \mathbf{j}}{\partial t^2} = a^2 \frac{\partial^2 \mathbf{j}}{\partial z^2}, \tag{10}$$

which demonstrate the indicated property.

Since at $t = 0$, $\omega = \mathbf{j} = 0$, it follows that

$$\mathbf{j} = \pm \frac{H_0}{2\pi a} \omega. \tag{11}$$

4. Propagation of z-Components of Vorticity and Current Density.—Equations (6) and (7), when projected on the $0z$ axis, give

$$2\rho_0 \frac{\partial \omega_z}{\partial t} = \mu_0 H_0 \frac{\partial j_z}{\partial z}, \tag{12}$$

$$2\pi \frac{\partial j_z}{\partial t} = H_0 \frac{\partial \omega_z}{\partial z}. \tag{13}$$

Hence

$$\frac{\partial^2 \omega_z}{\partial t^2} = a^2 \frac{\partial^2 \omega_z}{\partial z^2}, \tag{14}$$

$$\frac{\partial^2 j_z}{\partial t^2} = a^2 \frac{\partial^2 j_z}{\partial z^2}, \tag{15}$$

and

$$j_z = \pm \frac{H_0}{2\pi a} \omega_z. \tag{16}$$

Thus, the components of ω and $\mathbf{j}$ along the lines of force of the undisturbed magnetic field are propagated in the opposite direction of the field with velocities $\pm a$, in such a way that the vanishing of either quantity involves the vanishing of the other.

5. Equations for the Other Components.—We begin with the equations

$$\frac{\partial \omega_x}{\partial x} + \frac{\partial \omega_y}{\partial y} + \frac{\partial \omega_z}{\partial z} = 0, \tag{17}$$

$$\frac{\partial j_x}{\partial x} + \frac{\partial j_y}{\partial y} + \frac{\partial j_z}{\partial z} = 0. \tag{18}$$

The latter can be rewritten, by virtue of equation (16),

$$\frac{\partial j_x}{\partial x} + \frac{\partial j_y}{\partial y} \pm \frac{H_0}{2\pi a} \frac{\partial \omega_z}{\partial z} = 0. \tag{19}$$

Equations (17) and (19) indicate that

$$j_x = \pm \frac{H_0}{2\pi a} \omega_x + F(y, z, t), \qquad F(y, z, o) = 0; \tag{20), (21}$$

$$j_y = \pm \frac{H_0}{2\pi a} \omega_y + G(x, z, t), \qquad G(x, z, o) = 0; \tag{22), (23}$$

F and G being two arbitrary functions to be determined *a posteriori*. Substitution of results (20) and (22) into equations (6) and (7) yields

$$\frac{\partial \omega_x}{\partial t} \mp a \frac{\partial \omega_x}{\partial z} = \frac{2\pi a^2}{H_0} \frac{\partial F}{\partial z}, \tag{24}$$

$$\frac{\partial \omega_y}{\partial t} \mp a \frac{\partial \omega_y}{\partial z} = \frac{2\pi a^2}{H_0} \frac{\partial G}{\partial z}, \tag{25}$$

and

$$\frac{\partial F}{\partial t} \pm a \frac{\partial F}{\partial z} = \frac{H_0}{4\pi \rho_0} \frac{\partial^2 \rho}{\partial t \partial y}, \tag{26}$$

$$\frac{\partial G}{\partial t} \pm a \frac{\partial G}{\partial z} = -\frac{H_0}{4\pi \rho_0} \frac{\partial^2 \rho}{\partial t \partial x}. \tag{27}$$

Also, from equations (20) and (22)

$$\frac{\partial j_x}{\partial t} \mp a \frac{\partial j_x}{\partial z} = \frac{\partial F}{\partial t}, \tag{28}$$

$$\frac{\partial j_y}{\partial t} \mp a \frac{\partial j_y}{\partial z} = \frac{\partial G}{\partial t}. \tag{29}$$

Finally, combining equations (24)–(29) we obtain

$$\frac{\partial^2 \omega_x}{\partial t^2} - a^2 \frac{\partial^2 \omega_x}{\partial z^2} = \frac{a^2}{2\rho_0} \frac{\partial^3 \rho}{\partial t \partial y \partial z}, \tag{30}$$

$$\frac{\partial^2 \omega_y}{\partial t^2} - a^2 \frac{\partial^2 \omega_y}{\partial z^2} = -\frac{a^2}{2\rho_0} \frac{\partial^3 \rho}{\partial t \partial x \partial z} \tag{31}$$

and

$$\frac{\partial^2 j_x}{\partial t^2} - a^2 \frac{\partial^2 j_x}{\partial z^2} = \frac{H_0}{4\pi\rho_0} \frac{\partial^3 \rho}{\partial t^2 \partial y}, \tag{32}$$

$$\frac{\partial^2 j_y}{\partial t^2} - a^2 \frac{\partial^2 j_y}{\partial z^2} = -\frac{H_0}{4\pi\rho_0} \frac{\partial^3 \rho}{\partial t^2 \partial x} \tag{33}$$

Equations (30)–(33) could, of course, be derived directly from equations (6) and (7); the above analysis has, however, shown their fine structure. They yield, on elimination of ρ by cross-differentiation, equations (14) and (15).

It is of interest to note that for an incompressible material, or in the case when ρ does not depend on x and y, the solutions of equations (26) and (27) subject to initial conditions (21) and (23) are $F = G = 0$. This can easily be seen by taking their Laplace transform with respect to time. In these cases only, the coupling relationship between $\mathbf{j}$ and ω is identical to that resulting from Alfvén's theory. In general, therefore, *the vanishing of ω will leave a current density in planes perpendicular to $\mathbf{H}_0$ and vice-versa, as shown by formulas (20)–(23).*

The combination of equations (26) and (27) yields

$$\frac{\partial}{\partial y}\left(\frac{\partial F}{\partial t} \pm a \frac{\partial F}{\partial z}\right) - \frac{\partial}{\partial x}\left(\frac{\partial G}{\partial t} \pm a \frac{\partial G}{\partial z}\right) = \frac{H_0}{4\pi\rho_0} \frac{\partial}{\partial t}\left(\frac{\partial^2 \rho}{\partial x^2} + \frac{\partial^2 \rho}{\partial y^2}\right). \tag{34}$$

6. Equation for the Hydrodynamic Density.—Differentiation with respect to t of terms of equation (3) yields

$$\frac{\partial^2 \rho}{\partial t^2} = c^2 \nabla^2 \rho + \frac{a^2 \rho_0}{H_0} \nabla^2 h_z. \tag{35}$$

Now

$$\nabla^2 h_z = 4\pi\left(\frac{\partial j_x}{\partial y} - \frac{\partial j_y}{\partial x}\right). \tag{36}$$

Using equations (20) and (22) this can be rewritten

$$\nabla^2 h_z = \pm \frac{2H_0}{a}\left(\frac{\partial \omega_x}{\partial y} - \frac{\partial \omega_y}{\partial x}\right) + 4\pi\left(\frac{\partial F}{\partial y} - \frac{\partial G}{\partial x}\right). \tag{37}$$

Therefore,

$$\frac{\partial^2\rho}{\partial t^2} = c^2\nabla^2\rho \pm 2a\rho_0\left(\frac{\partial\omega_x}{\partial y} - \frac{\partial\omega_y}{\partial x}\right) + \frac{4\pi a^2\rho_0}{H_0}\left(\frac{\partial F}{\partial y} - \frac{\partial G}{\partial x}\right). \tag{38}$$

This result may be contrasted with the wave equation for ρ encountered in acoustics and is in agreement with it, if we let $H_0 = 0$.

7. *The Case When $\omega_x = \omega_y = 0$.*—We can now imagine motions in which the vorticity lies entirely along the magnetic field $\mathbf{H}_0$. They are of a considerable practical interest since they can be illustrated by simple mechanical lattices. In this case our equations will be simplified greatly. Equations (24) and (25) give

$$\frac{\partial F}{\partial z} = \frac{\partial G}{\partial z} = 0. \tag{39}$$

Hence, equation (34) reduces to

$$\frac{\partial}{\partial t}\left(\frac{\partial F}{\partial y} - \frac{\partial G}{\partial x}\right) = \frac{H_0}{4\pi\rho_0}\frac{\partial}{\partial t}\left(\frac{\partial^2\rho}{\partial x^2} + \frac{\partial^2\rho}{\partial y^2}\right). \tag{40}$$

Recalling that before the passage of the wave there were no perturbations, this gives

$$\frac{\partial F}{\partial y} - \frac{\partial G}{\partial x} = \frac{H_0}{4\pi\rho_0}\left(\frac{\partial^2\rho}{\partial x^2} + \frac{\partial^2\rho}{\partial y^2}\right), \tag{41}$$

and equation (38) becomes

$$\frac{\partial^2\rho}{\partial t^2} = c^2\nabla^2\rho + a^2\left(\frac{\partial^2\rho}{\partial x^2} + \frac{\partial^2\rho}{\partial y^2}\right). \tag{42}$$

Let us reconsider equation (14), where the subscript z can now be suppressed. The equation now describes the wave motion of vorticity ω (in opposite directions) of the undisturbed magnetic field. This motion is accompanied by a current density vector whose z-component is coupled with vorticity by equation (16) and x, y components (in planes perpendicular to $\mathbf{H}_0$) are given by

$$j_x = F(y, t), \tag{43}$$

$$j_y = G(x, t). \tag{44}$$

8. *A Simple Mechanical Model for Vorticity Propagation.*—In order to give a physical picture of this wave-motion, consider a chain of n particles along a line of force; suppose that the lowest article suddenly spins around the magnetic field. According to (16) an electric current is induced that interacts with the next neighboring particle and sets it in rotation. The rotation of this particle, in turn, induces a new current that is sent to the next neighbor, and so on. The following question now arises: Is the disturbance going to reach the nth highest particle? To answer this, we replace, as is usual in numerical integration, equation (14) by

$$\rho_0\ddot{\omega}_p = k(\omega_{p-1} - 2\omega_p + \omega_{p+1}) \tag{45}$$

where $k = (\mu_0/4\pi)(H_0/d)^2$, in which d is the infinitesimal distance between two par-

ticles, ω_0 and ω_{n+1} are the extreme particles in the chain (whose motion must be prescribed), and $p = 1, 2, \ldots,, n$. Supposing that

$$\omega_0 = Ae^{i\alpha t}, \qquad \omega_{n+1} = 0, \tag{46), (47}$$

we may try solutions of (45) of the type

$$\omega_p = A_p e^{i(\alpha t - \beta)}. \tag{48}$$

We now follow *mutatis mutandis* the example given by Brillouin and Parodi.[6] Setting

$$2\zeta = -2 + (\rho_0/k)\alpha^2, \tag{49}$$

we have

$$\left.\begin{aligned} &2\zeta A_1 + A_2 = -A^{i\beta}, \\ &\cdots\cdots\cdots\cdots\cdots \\ &A_{p-1} + 2\zeta A_p + A_{p+1} = 0, \\ &\cdots\cdots\cdots\cdots\cdots \\ &A_{n-1} + 2\zeta A_n = 0. \end{aligned}\right\} \tag{50}$$

The amplitude of the motion of the nth particle in the chain can readily be obtained:

$$A_n = \frac{(-1)^n A}{C_n^1(\zeta)}\, e^{i\beta} \tag{51}$$

where $C_n^1(\zeta)$ is Gegenbauer's electrospherical polynomial of order n. It thus appears that there is a sharp distinction in the conditions of motion of the nth particle according to the magnitude of $|\zeta|$, provided that the number of particles is sufficiently large and k is finite. If $|\zeta| < 1$, that is $0 < \alpha < 2\sqrt{k/\rho_0}$, there is propagation; on the contrary if $|\zeta| > 1$, that is $\alpha > 2\sqrt{k/\rho_0}$, then $|A_n| \to 0$ and there is attenuation and no propagation. The structure considered behaves, therefore, like a low-pass filter.

This treatment can, of course, be extended to more complicated lattices; as models for such analysis the reader is referred to the classical studies of Brillouin and Parodi.

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THE HYDRODYNAMIC STABILITY OF INVISCID FLOW BETWEEN COAXIAL CYLINDERS*

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1. It is known that the equations of inviscid flow of an incompressible fluid allow as stationary solutions an arbitrary distribution of angular velocity, $\Omega\ (r)$, about an axis (where r is the distance from the axis). Rayleigh's criterion for the stability of such flow is

$$\Phi(r) = 2\frac{\Omega}{r}\frac{d}{dr}(r^2\Omega) > 0. \tag{1}$$

When viscosity is present, the class of permissible flows becomes very restricted and the most general form of $\Omega(r)$ which is allowed is

$$\Omega(r) = A + B/r^2, \tag{2}$$

where A and B are two constants which are related to the angular velocities Ω_1 and Ω_2 with which the inner and the outer cylinders are rotated. Rayleigh's criterion for stability when applied to the distribution (2) leads to the condition

$$\Omega_2/\Omega_1 > (R_1/R_2)^2 \tag{3}$$

where R_1 and R_2 are, respectively, the radii of the inner and the outer cylinders. The presence of viscosity inhibits the onset of instability and condition (3) must be violated by a certain determinate extent before instability can arise. The extent of this inhibition has been considered by a number of authors since G. I. Taylor first investigated the problem.[1]

The equations of viscous flow allow stationary solutions besides those given by (2). Thus a constant transverse pressure gradient, $\partial p/r\partial\theta$, leads to a superposition of a Poiseuille type of flow on the distribution (2). The stability of such flows has been considered by Dean[2], Reid[3], and DiPrima,[4] among others. Another type of generalization is the superposition of an axial flow over (2). The stability of such non-Couette flows has been considered by Goldstein,[5] but Goldstein's treatment was of a preliminary character.[6] In undertaking a more comprehensive treatment of the latter problem, it became clear that the corresponding problem in inviscid flow has not been considered. The present paper is devoted to its consideration.

2. By writing the equations of inviscid flow in cylindrical polar-coordinates, (r, θ, z), we can directly verify that

$$u_r = 0, \qquad u_\theta = V(r), \qquad u_z = W(r), \tag{4}$$

and

$$\frac{1}{\rho}\frac{dp}{dr} = \frac{V^2}{r}, \tag{5}$$

where $V\ (r)$ and $W\ (r)$ are arbitrary functions of r, represent a stationary solution

of the equations. By considering an infinitesimal perturbation of the foregoing solution, we can readily obtain the required linear equations governing it. For axisymmetric perturbations (to which we shall restrict ourselves), the equations are

$$\frac{\partial u}{\partial t} + W\frac{\partial u}{\partial z} - 2\frac{V}{r}v = -\frac{\partial \varpi}{\partial r}, \tag{6}$$

$$\frac{\partial v}{\partial t} + W\frac{\partial v}{\partial z} + \left(\frac{dV}{dr} + \frac{V}{r}\right)u = 0, \tag{7}$$

$$\frac{\partial w}{\partial t} + W\frac{\partial w}{\partial z} + u\frac{dW}{dr} = -\frac{\partial \varpi}{\partial z}, \tag{8}$$

where u, v, and w are the components of the perturbed velocity along the three principal directions and ϖ ($= \delta p/\rho$) is proportional to the perturbation in the pressure. In addition to equations (6)–(8), we have the equation of continuity (again, for axisymmetric motions)

$$\frac{\partial u}{\partial r} + \frac{u}{r} + \frac{\partial w}{\partial z} = 0. \tag{9}$$

3. We seek solutions of equations (6)–(9) whose dependence on t and z is given by

$$e^{i(pt + kz)} \tag{10}$$

where k denotes the wave number of the disturbance. The discussion of stability centers on the reality or otherwise of p. For solutions having the chosen (z, t)-dependence, the perturbation equations become

$$i(p + kW)u - 2\Omega v = -D\varpi, \tag{11}$$

$$i(p + kW)v = -(D_*V)u. \tag{12}$$

$$i(p + kW)w + (DW)u = -ik\varpi, \tag{13}$$

and

$$D_*u = -ikw, \tag{14}$$

where u, v, and w are now only functions of r and

$$D = d/dr \text{ and } D_* = D + 1/r. \tag{15}$$

Eliminating w between equations (13) and (14), we obtain

$$(p + kW)D_*u - (kDW)u = ik^2\varpi. \tag{16}$$

Differentiating this equation with respect to r and making use of equations (11), we obtain

$$D[(p + kW)D_*u] - D[(kDW)u] = -ik^2[i(p + kW)u - 2\Omega v]. \tag{17}$$

On simplification, this equation becomes

$$(p + kW)(DD_* - k^2)u - k\Psi(r)u = 2ik^2\Omega v, \tag{18}$$

where

$$\Psi(r) = r \frac{d}{dr}\left(\frac{1}{r}\frac{dW}{dr}\right). \tag{19}$$

Substituting for v from equation (12) in (18), we finally obtain

$$(p + kW)\,(DD_* - k^2)\,u - k\Psi(r)u = -k^2\Phi(r)\,\frac{u}{p + kW}, \tag{20}$$

where

$$\Phi(r) = 2\Omega D_* V = 2\frac{\Omega}{r}\frac{d}{dr}(rV) = 2\frac{\Omega}{r}\frac{d}{dr}(r^2\Omega) \tag{21}$$

is Rayleigh's discriminant (1) as previously defined. The solution of equation (20) must be sought which satisfies the boundary conditions

$$u = 0 \text{ for } r = R_1 \text{ and } R_2. \tag{22}$$

4. An alternative form of equation (20) which we shall find useful is obtained by the substitution

$$i\chi = \frac{u}{p + kW}. \tag{23}$$

This substitution is permissible in virtue of equation (12) according to which

$$v = -(D_* V)\chi. \tag{24}$$

In terms of χ, equation (16) takes the form

$$(p + kW)^2\, D_*\chi = k^2\varpi, \tag{25}$$

while equation (11) becomes

$$(p + kW)^2\chi - \Phi(r)\,\chi = D\varpi. \tag{26}$$

Now eliminating ϖ between equations (25) and (26), we obtain

$$D[(p + kW)^2 D_*\chi] - k^2(p + kW)^2\,\chi = -k^2\Phi(r)\chi. \tag{27}$$

And the boundary conditions with respect to which this equation must be solved are (cf. eq. [23])

$$\chi = 0 \qquad \text{for } r = R_1 \text{ and } R_2. \tag{28}$$

5. In the case of a purely axial flow, equation (20) reduces to

$$(DD_* - k^2)\,u - k\Psi(r)\,\frac{u}{p + kW} = 0. \tag{29}$$

Multiplying this equation by ru^* and integrating over the range of r we obtain (after one partial integration)

$$\int r\,\{|D_* u|^2 + k^2|u|^2\}\,dr + k\int \frac{\Psi(r)\,r|u|^2}{p + kW}\,dr = 0. \tag{30}$$

The vanishing of the imaginary part of this equation requires

$$\Im(p) \int \frac{\Psi(r)\, r|u|^2}{|p + kW|^2}\, dr = 0. \tag{31}$$

If $\Im(p) \neq 0$, a necessary condition for the validity of equation (31) is $\Psi(r)$ changes sign in the interval. If $\Psi(r)$ is of one sign, $J(p) = 0$ and the flow must be stable. The condition that for instability $\Psi(r)$ must change sign is the present analogue of Rayleigh's condition that for instability of plane-parallel flow, there should be a point of inflexion in the velocity profile. And the usual analysis[7] for plane-Poiseuille flow can be repeated with respect to equation (29).

6. Returning to the general case and writing

$$\Omega = \Omega_1\, \omega(r), \tag{32}$$

where Ω_1 is the angular velocity at $r = R_1$ (say), we can consider equation (27) together with the boundary conditions (28) as constituting a characteristic value problem for Ω_1^2. Thus, letting

$$\phi(r) = 2\frac{\omega}{r}\frac{d}{dr}(r^2\omega) \qquad \text{and} \qquad c = \frac{p}{k}, \tag{33}$$

we have

$$D[(W + c)^2 D_* \chi] - k^2(W + c)^2 \chi = -\Omega_1^2 \phi(r)\, \chi. \tag{34}$$

Considered as a characteristic value problem for Ω_1^2 (for given c and k, both assumed real and $c < -W_{\max}$ or $> -W_{\min}$), the standard theorems of the classical Sturmian theory apply. We have: the characteristic values of Ω_1^2 are all positive if $\phi(r)$ is everywhere positive and they are all negative if $\phi(r)$ is everywhere negative, while if $\phi(r)$ takes different signs in the allowed range of r, there are two sequences of characteristic values for Ω_1^2, a positive sequence and a negative sequence. Starting from these premises one can show (by arguments into which we shall not enter) that Rayleigh's criterion for stability continues to be valid irrespective of W. Thus, the presence of rotation alters the problem in a qualitative way: its orgin is rooted in equation (12) which couples the radial and the transverse perturbations in the velocity.

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[1] Taylor, G. I., "Stability of a Viscous Liquid Contained Between Two Rotating Cylinders," *Philos. Trans. Roy. Soc. London, A*, **223**, 289–343 (1923). For references to later work see Chandrasekhar, S., "The Stability of Viscous Flow Between Rotating Cylinders," *Mathematika*, **1**, 5–13 (1954).

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[5] Goldstein, S., "The Stability of Viscous Fluid Flow Between Rotating Cylinders," *Proc. Camb. Philos. Soc.*, **33**, 41–61 (1937).

[6] The calculations undertaken by the writer do not confirm Goldstein's specific results; in particular, the sharp decrease in the critical Taylor number which he finds when the Reynolds number of the axial flow exceeds 20. Goldstein's results are, moreover, inconsistent with the general theorem proved in §6.

[7] Lin, C. C., *The Theory of Hydrodynamic Stability* (Cambridge: Cambridge University Press, 1955), §8.2.

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*THE HYDRODYNAMIC STABILITY OF VISCID FLOW BETWEEN COAXIAL CYLINDERS**

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Communicated November 27, 1959

1. The stability of viscous flow between two rotating coaxial cylinders when a constant pressure gradient is acting along the axis has been considered by Goldstein.[1] Goldstein's calculations seemed to indicate that when the Reynolds number of the axial flow increases beyond a certain relatively small value ($\sim$25), the flow has the effect of destabilizing the rotational flow: indeed, a little extrapolation of his results would actually suggest that an axial flow can destabilize even a stable rotational flow. This latter conclusion is contrary to the results of the preceding paper[2] according to which, for inviscid flow, the presence of an axial flow does not affect Rayleigh's criterion. For this reason calculations were undertaken to solve the underlying characteristic value problem by a method which has proved successful in other connections[3]; and the new calculations do not confirm Goldstein's results.

2. If we restrict ourselves to the case when the gap, $d = R_2 - R_1$, between the two cylinders is small compared to their mean radius, $\frac{1}{2}(R_2 + R_1)$, the initial stationary flow can be approximated by a distribution of angular velocity

$$\Omega_1[1 - (1 - \mu)\zeta], \tag{1}$$

together with an axial flow

$$6V_m\zeta(1 - \zeta), \tag{2}$$

where Ω_1 and Ω_2 are the angular velocities of the inner and the outer cylinders, V_m is the mean axial flow,

$$\zeta = (r - R_1)/(R_2 - R_1) \text{ and } \mu = \Omega_2/\Omega_1. \tag{3}$$

3. By considering an infinitesimal perturbation of the flow represented by equations (1) and (2), and analyzing the disturbance into normal modes by seeking solutions whose (z, t)—dependence is given by

$$e^{i(pt + kz)}, \tag{4}$$

we readily obtain the following equations[4]

$$\{(D^2 - a^2) - i[\sigma + 6Ra(\tfrac{1}{4} - \zeta^2)]\}(D^2 - a^2)u - 12iRau = v\left(1 - 2\frac{1 - \mu}{1 + \mu}\zeta\right), \tag{5}$$

and

$$\{(D^2 - a^2) - i[\sigma + 6Ra(1/4 - \zeta^2)]\}v = -Ta^2u, \tag{6}$$

where $$\sigma = pd^2/\nu$$

and $$a = kd. \tag{7}$$

Also $$R = V_m d/\nu \tag{8}$$

is the Reynolds number associated with the mean axial flow,

$$T = -2\Omega_1 A(1 + \mu)d^4/\nu^2 \tag{9}$$

is the Taylor number, and

$$A = \Omega_1 \frac{1 - \mu R_2^2/R_1^2}{1 - R_2^2/R_1^2}, \tag{10}$$

[In the foregoing equations the origin of ζ has been displaced to be midway between R_1 and R_2.]

Solutions of equations (5) and (6) must be sought which satisfy the boundary conditions

$$u = Du = v = 0 \quad \text{for} \quad \zeta = \pm 1/2. \tag{11}$$

4. Equations (5) and (6) together with the boundary conditions (11) constitute a characteristic value problem: for a given a, R, and σ the Taylor number, T, can have only one of a sequence of possible determinate values. For an assigned real value of σ, the characteristic values of T will in general, be complex. The requirement that T be real will determine σ; and the critical Taylor number for the onset of instability (for a given R) will be given by the minimum of the real characteristic values of T so determined and considered as a function of a.

5. For $\mu > 0$, it is known that in case $R = 0$, the term linear in ζ on the right-hand side of equation (5) affects the deduced values of T only to the second order in $(1 - \mu)/(1 + \mu)$ and contributes a correction of the order of one per cent. On this account, it will appear that we may replace in equations (5) and (6) the terms which allow for the variation of $\Omega(\zeta)$ and $W(\zeta)$ across the gap by their respective averages. Thus we replace equations (5) and (6) by

$$\{[D^2 - a^2 - i(\sigma + Ra)](D^2 - a^2) - 12iRa\}u = v \tag{12}$$

and

$$[D^2 - a^2 - i(\sigma + Ra)]v = -Ta^2u. \tag{13}$$

6. The characteristic value problem presented by equations (11)–(13) can be solved conveniently by the method described by the author for the solution of the original Taylor problem.[3] We shall not give the details of the analysis but quote only the results of the calculations. These are given in the adjoining table.

TABLE 1

THE CRITICAL TAYLOR NUMBERS AND RELATED CONSTANTS FOR DIFFERENT REYNOLDS NUMBERS

R	a	T	σ
0	3.1	1715	0
5	3.1	1753	12.6
20	3.4	2309	55.7
40	4.2	3881	140.9
60	5.2	5962	270.2
80	6.0	8319	425
100	6.6	10876	594

It will be observed that contrary to Goldstein's results, T appears as a monotonic increasing function of R.

After the calculations presented here were almost completed, the author learned that the same problem has been considered by Dr. R. DiPrima with very similar results.

I am greatly indebted to Miss Donna Elbert for carrying out the numerical solutions.

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[1] Goldstein, S., "The Stability of Viscous Fluid Flow between Rotating Cylinders," *Proc. Camb. Philos. Soc.*, **33**, 41–61 (1937).

[2] Chandrasekhar, S., "The Hydrodynamic Stability of Inviscid Flow between Coaxial Cylinders," these PROCEEDINGS, 137–141 (1960).

[3] Chandrasekhar, S., "The Stability of Viscous Flow between Rotating Cylinders," *Mathematika*, **1**, 5–13 (1954).

[4] Equations (5) and (6) are equivalent to equations (25) and (27) of Goldstein's paper (see reference 1 above).

ON THE RELATION BETWEEN REPRODUCTIVE VALUE AND OPTIMAL PREDATION

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An important problem of ecology is to determine at what rate individuals should be removed ("harvested") from a population so as to achieve the greatest continued yield. This is of obvious practical importance in agriculture, fisheries, and forestry and is of theoretical importance in understanding how a predator should operate. Watt[1] and Beverton and Holt[2] and Slobodkin[3] have given some practical solutions to the problem, but the approach used here is very concise and gives different insight into the theoretical aspects.

The answer will be given in two stages. First, consider the stable situation in which individuals are removed from the population independently of age at a constant rate such that the population size and composition also remain constant.

Let the probability density that an individual is removed be r. Let n_x be the density of individuals of age x in the population so that $\int_0^\infty n_x\,dx = N$ is the total number of individuals present. Let l_x and b_x be survivorship and birth rates at age x, in the absence of predation. The actual chance of an individual surviving to age x is then given by $e^{-rx}l_x$, and therefore $n_x = n_0\,e^{-rx}l_x$. Thus the (constant) number of newborn is given by

$$n_0 = \int_0^\infty n_x b_x dx = n_0 \int_0^\infty e^{-rx}\, l_x b_x dx \qquad \text{so that}$$

$$1 = \int_0^\infty e^{-rx} l_x v_x dx. \tag{1}$$

This is familiar as the equation determining the "intrinsic rate of natural increase" r and shows that the rate of predation which keeps the population constant is the r that the ecologist uses regularly. Now there is in general a different value of r for each population size and composition. The problem of optimal predation, independent of age, is then solved by finding that feasible age distribution for which rN, the total harvest, is a maximum. This much is quite obvious.

Conversely, if a population is kept in a constant composition by predation, the rate of removal equals the intrinsic rate of natural increase. This may be the most feasible way of measuring r for some species.

Frequently, however, the age of organisms can be determined, so that the organisms of different ages can be removed at different rates. The question now becomes "At what rate should organisms of age x be removed to maximize the yield?" In 1929, R. A. Fisher[4] proposed a very ingenious definition of the "reproductive value" of an individual or a population which makes possible a very similar solution of this problem. The reproductive value v_x of an individual of age x is defined by

$$v_x = \frac{e^{rx}}{l_x} \int_0^\infty e^{-rx}\, l_x b_x dx$$

and the reproductive value V of the whole population is obtained by integrating over all its individuals:

$$V = \int_0^\infty n_x v_x dx$$

Fisher showed that, no matter what the age distribution,

$$\frac{dV}{dt} = rV \tag{2}$$

where r, as before, is the solution of (1). Notice that r varies with population composition as l_x, b_x vary. However, for each population composition (i.e., each set of values of n_x) both r and V are well defined and equation (2) holds. This means that, if one harvests a population at the rate rV, then $dv/dt = 0$, i.e., its reproductive value will not change, even though its age composition may change greatly. If now we make the assumption (probably nearly true in most cases) that

different age distributions which do not differ in total reproductive value V also do not differ in r, then a complete solution of the problem is available. For on removing individuals at a rate rV, the reproductive value V and, now, also r are left unchanged so that this rate of removal can be continued indefinitely (in spite of the fact that the age distribution may be changing continuously). This removal is specified in terms not of individuals of age x but of reproductive value. The same reproductive value may be removed by taking few individuals of high reproductive value or many individuals of low.

If all ages are of equal value to the harvester, individuals with the lowest reproductive value should be removed first. Fisher's term "reproductive value" is sufficiently well chosen that this conclusion seems almost intuitive and should thus be easy to remember. As a practical conclusion, post-reproductive ages (with zero reproductive value) should normally be removed first. However, in many natural populations the expectations of further life and the reproductive rate do not fall off appreciably with age[5] so that reproductive value remains high during adult life. For these species, the reproductive value is normally lowest for the newborn who will be subject to heavy mortality before reaching sexual maturity, and a heavy removal of newborn individuals would be the most efficient predation in the sense of removing individuals at the greatest rate.

In such species as fish, however, newborn are of much less value than mature individuals to the harvester or predator. For these species optimal predation consists in removing individuals of ages for which the ratio (value to predator/reproductive value) is greatest. The total rate at which value to predator is accumulated is then rV times (value to predator/reproductive value).

In summary, a predator that cannot tell the age of its prey should adjust the prey population size N so that rN is a maximum and then remove individuals at this rate. A predator that can remove different ages of prey at different rates should adjust the prey population so that r times its reproductive value is a maximum and remove individuals at this rate, taking ages with the largest ratio (value to predator/reproductive value).

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